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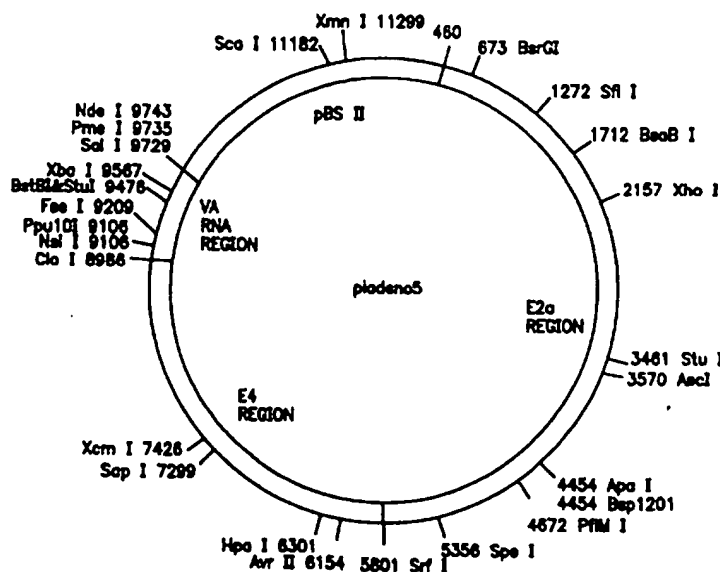
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(54) Title: ACCESSORY FUNCTIONS FOR USE IN RECOMBINANT AAV VIRION PRODUCTION



## (57) Abstract

Accessory functions capable of supporting efficient recombinant AAV (rAAV) virion production in a suitable host cell are provided. The accessory functions are in the form of one or more vectors comprising a nucleotide sequence selected from the group consisting of (i) a sequence that provides adenovirus VA RNAs, (ii) an adenovirus E4 ORF6 coding region, (iii) an adenovirus E2a 72 kD coding region, and any combination of nucleotide sequences (i), (ii) and (iii), and that are capable of being transferred between cells. Methods of producing rAAV virions are also provided. The methods can be practised to produce commercially significant levels of rAAV particles without also generating significant levels of infectious helper virus or other contaminating by-products.

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ACCESSORY FUNCTIONS FOR USE IN  
RECOMBINANT AAV VIRION PRODUCTION

5

Technical Field

The present invention relates generally to  
10 accessory functions for use in adeno-associated virus  
(AAV) virion production. More particularly, the  
invention relates to vectors which provide accessory  
functions capable of supporting efficient recombinant  
AAV virion production in a suitable host cell, and  
15 methods of use thereof.

Background of the Invention

Gene delivery is a promising method for the  
treatment of acquired and inherited diseases. A  
20 number of viral based systems for gene transfer  
purposes have been described, such as retroviral  
systems which are currently the most widely used viral  
vector systems for this purpose. For descriptions of  
various retroviral systems, see, e.g., U.S. Patent No.  
25 5,219,740; Miller and Rosman (1989) *BioTechniques*  
7:980-990; Miller, A.D. (1990) *Human Gene Therapy* 1:5-  
14; Scarpa et al. (1991) *Virology* 180:849-852; Burns  
et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:8033-8037;  
and Boris-Lawrie and Temin (1993) *Cur. Opin. Genet.*  
30 *Develop.* 3:102-109.

Adeno-associated virus (AAV) systems have  
also been used for gene delivery. AAV is a helper-  
dependent DNA parvovirus which belongs to the genus  
*Dependovirus*. AAV requires infection with an  
35 unrelated helper virus, either adenovirus, a  
herpesvirus or vaccinia, in order for a productive  
infection to occur. The helper virus supplies

accessory functions that are necessary for most steps in AAV replication. In the absence of such infection, AAV establishes a latent state by insertion of its genome into a host cell chromosome. Subsequent  
5 infection by a helper virus rescues the integrated copy which can then replicate to produce infectious viral progeny. AAV has a wide host range and is able to replicate in cells from any species so long as there is also a successful infection of such cells  
10 with a suitable helper virus. Thus, for example, human AAV will replicate in canine cells co-infected with a canine adenovirus. AAV has not been associated with any human or animal disease and does not appear to alter the biological properties of the host cell  
15 upon integration. For a review of AAV, see, e.g., Berns and Bohenzky (1987) *Advances in Virus Research* (Academic Press, Inc.) 32:243-307.

The AAV genome is composed of a linear, single-stranded DNA molecule which contains 4681 bases  
20 (Berns and Bohenzky, *supra*). The genome includes inverted terminal repeats (ITRs) at each end which function in *cis* as origins of DNA replication and as packaging signals for the virus. The ITRs are approximately 145 bp in length. The internal  
25 nonrepeated portion of the genome includes two large open reading frames, known as the AAV *rep* and *cap* regions, respectively. These regions code for the viral proteins involved in replication and packaging of the virion. In particular, a family of at least  
30 four viral proteins are synthesized from the AAV *rep* region, Rep 78, Rep 68, Rep 52 and Rep 40, named according to their apparent molecular weight. The AAV *cap* region encodes at least three proteins, VP1, VP2 and VP3. For a detailed description of the AAV  
35 genome, see, e.g., Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129.

The construction of recombinant AAV virions has been described. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Numbers WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al. (1988) *Molec. Cell. Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines 90* (Cold Spring Harbor Laboratory Press); Carter, B.J. (1992) *Current Opinion in Biotechnology* 3:533-539; Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; and Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801.

Contemporary recombinant AAV (rAAV) virion production involves co-transfection of a host cell with an AAV vector plasmid and a construct which provides AAV helper functions to complement functions missing from the AAV vector plasmid. In this manner, the host cell is capable of expressing the AAV proteins necessary for AAV replication and packaging. The host cell is then infected with a helper virus to provide accessory functions. The helper virus is generally an infectious adenovirus (type 2 or 5), or herpesvirus.

AAV helper functions can be provided via an AAV helper plasmid that includes the AAV *rep* and/or *cap* coding regions but which lacks the AAV ITRs. Accordingly, the helper plasmid can neither replicate nor package itself. A number of vectors that contain the *rep* coding region are known, including those vectors described in U.S. Patent No. 5,139,941, having ATCC Accession Numbers 53222, 53223, 53224, 53225 and 53226. Similarly, methods of obtaining vectors containing the HHV-6 homologue of AAV *rep* are described in Thomson et al. (1994) *Virology* 204:304-311. A number of vectors containing the *cap* coding region have also been described, including those vectors described in U.S. Patent No. 5,139,941.

AAV vector plasmids can be engineered to contain a functionally relevant nucleotide sequence of interest (e.g., a selected gene, antisense nucleic acid molecule, ribozyme, or the like) that is flanked  
5 by AAV ITRs which provide for AAV replication and packaging functions. After an AAV helper plasmid and an AAV vector plasmid bearing the nucleotide sequence are introduced into the host cell by transient transfection, the transfected cells can be infected  
10 with a helper virus, most typically an adenovirus, which, among other functions, transactivates the AAV promoters present on the helper plasmid that direct the transcription and translation of AAV rep and cap regions. Upon subsequent culture of the host cells,  
15 rAAV virions (harboring the nucleotide sequence of interest) and helper virus particles are produced.

When the host cell is harvested and a crude extract is produced, the resulting preparation will contain, among other components, approximately equal  
20 numbers of rAAV virion particles and infectious helper virions. rAAV virion particles can be purified away from most of the contaminating helper virus, unassembled viral proteins (from the helper virus and AAV capsid) and host cell proteins using known  
25 techniques. Purified rAAV virion preparations that have been produced using infection with adenovirus type-2 contain high levels of contaminants. Particularly, 50% or greater of the total protein obtained in such rAAV virion preparations is free  
30 adenovirus fiber protein. Varying amounts of several unidentified adenoviral and host cell proteins are also present. Additionally, significant levels of infectious adenovirus virions are obtained, necessitating heat inactivation. The contaminating  
35 infectious adenovirus can be inactivated by heat treatment (56°C for 1 hour) and rendered undetectable by sensitive adenovirus growth assays (e.g., by

cytopathic effect (CPE) in a permissive cell line). However, heat treatment also results in an approximately 50% drop in the titer of functional rAAV virions.

5           Production of rAAV virions using an infectious helper virus (such as an adenovirus type-2, or a herpesvirus) to supply accessory functions is undesirable for several reasons. AAV vector production methods which employ a helper virus require  
10 the use and manipulation of large amounts of high titer infectious helper virus which presents a number of health and safety concerns, particularly in regard to the use of a herpesvirus. Also, concomitant production of helper virus particles in rAAV virion  
15 producing cells diverts large amounts of cellular resources away from rAAV virion production, possibly resulting in lower rAAV virion yields.

          More particularly, in methods where infection of cells with adenovirus type-2 are used to  
20 provide the accessory functions, more than 95% of the contaminants found in the purified rAAV virion preparations are derived from adenovirus. The major contaminant, free adenovirus fiber protein, tends to co-purify with rAAV virions on CsCl density gradients  
25 due to a non-covalent association between the protein and rAAV virions. This association makes separation of the two especially difficult, lowering rAAV virion purification efficiency. Such contaminants may be particularly problematic since many adenoviral  
30 proteins, including the fiber protein, have been shown to be cytotoxic (usually at high concentrations), and thus may adversely affect or kill target cells. Thus, a method of producing rAAV virions without the use of infectious helper viruses to provide necessary  
35 accessory functions would be advantageous.

          A number of researchers have investigated the genetic basis of accessory functions, particularly



adenovirus-derived functions. Generally, two approaches have been used to attempt to identify those adenoviral genes that are involved in AAV replication: examination of the ability of various adenovirus mutants to provide accessory functions; and the study of the effect of transfected adenoviral genes or regions on AAV replication in the absence of adenovirus infection.

Studies with various adenovirus mutants that are capable of supporting AAV replication (e.g., by supplying necessary accessory functions) at or about the levels obtained by infection with a wild-type adenovirus have demonstrated that particular adenovirus genes or gene regions are not involved in AAV replication. However, loss-of-function data from such studies have failed to provide conclusive information that a particular gene region is involved with AAV replication since many of the adenovirus genes and control regions are overlapping and/or incompletely mapped.

Particularly, adenovirus mutants with fairly well characterized mutations in the following genes or gene regions have been tested for their ability to provide accessory functions necessary for AAV viral replication: E1a (Laughlin et al. (1982) *J. Virol.* 41:868, Janik et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:1925); E1b (Laughlin et al. (1982), *supra*, Janik et al. (1981), *supra*, Ostrove et al. (1980) *Virology* 104:502); E2a (Handa et al. (1975) *J. Gen. Virol.* 29:239, Straus et al. (1976) *J. Virol.* 17:140, Myers et al. (1980) *J. Virol.* 35:665, Jay et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:2927, Myers et al. (1981) *J. Biol. Chem.* 256:567); E2b (Carter, B.J. (1990) "Adeno-Associated Virus Helper Functions," in *CRC Handbook of Parvoviruses*, vol. I (P. Tijssen, ed.); E3 (Carter et al. (1983) *Virology* 126:505); and E4 (Carter et al. (1983), *supra*, Carter, B.J. (1995),

supra). Poorly characterized adenovirus mutants that were incapable of DNA replication and late gene synthesis have also been tested (Ito et al. (1970) *J. Gen. Virol.* 9:243, Ishibashi et al. (1971) *Virology* 5 45:317).

Adenovirus mutants with defects in the E2b and E3 regions have been shown to support AAV replication, indicating that the E2b and E3 regions are probably not involved in providing accessory functions (Carter et al. (1983), *supra*). Mutant adenoviruses defective in the E1a region, or having a deleted E4 region, are unable to support AAV replication, indicating that the E1a and E4 regions are likely required for AAV replication, either directly or indirectly (Laughlin et al. (1982), *supra*, Janik et al. (1981), *supra*, Carter et al. (1983), *supra*). Studies with E1b and E2a mutants have produced conflicting results. Further, adenovirus mutants incapable of DNA replication and late gene synthesis have been shown to be permissive for AAV replication (Ito et al. (1970), *supra*, Ishibashi et al. (1971), *supra*). These results indicate that neither adenoviral DNA replication nor adenoviral late genes are required for AAV replication.

Transfection studies with selected adenoviral genes have been used in an attempt to establish whether a transfected set of adenovirus genes is capable of providing the same level of accessory functions for AAV replication as that provided by an adenovirus infection. Particularly, *in vitro* AAV replication has been assessed using human 293 cells transiently transfected with various combinations of adenovirus restriction fragments encoding single adenovirus genes or groups of genes (Janik et al. (1981), *supra*). Since the above-described transfection studies were done in cells that stably express the adenovirus E1a and E1b regions, the

requirement for those regions could not be tested. However, it was deduced that the combination of three adenoviral gene regions, VA I RNA, E2a and E4, could provide accessory functions (e.g., support AAV  
5 replication) at a level that was substantially above background, but that was still approximately 8,000 fold below the level provided by infection with adenovirus. When all combinations of two of the three genes were tested, the accessory function levels  
10 ranged between 10,000 to 100,000 fold below the levels provided by infection with adenovirus.

Transfection studies with selected herpes simplex virus type-1 (HSV-1) genes have also been conducted in an attempt to establish whether a  
15 transfected set of HSV-1 genes is capable of providing the same level of accessory functions for AAV replication as that provided by an HSV-1 infection. Weindler et al. (1991) *J. Virol.* 65:2476-2483. However, such studies were limited to identifying only  
20 those HSV-1 genes necessary to support wild-type AAV replication, not rAAV production. Further, the identified HSV-1 accessory functions were significantly less efficient at supporting AAV replication than adenovirus-derived functions.

25 Accordingly, there remains a need in the art to identify a subset of the adenovirus genome or functional homologues of the adenovirus genome, that include only those accessory functions required for rAAV virion production. The subset can then be  
30 included in an accessory function vector or system which, when introduced into a suitable host cell, supports the production of rAAV virions in an amount that is substantially equivalent to, or greater than, the amount produced using an adenovirus infection.  
35 Further, there remains a need to provide an accessory function system that is capable of producing commercially significant levels of rAAV virion

particles without also generating significant levels of infectious adenovirus virions, or other contaminating by-products.

5    Summary of the Invention

          The present invention is based on the identification of the accessory functions needed to support efficient AAV replication in a suitable host cell. The invention provides a system which includes  
10   such functions and allows for the production of rAAV virions without the use of a helper virus.

          In certain embodiments, the invention relates to nucleic acid molecules encoding accessory functions and that lack at least one adenoviral late  
15   gene region. The molecules can be provided in one or more vectors which include nucleotide sequences derived from an adenovirus type-2 or type-5 genome, or functional homologues thereof. Thus, in one aspect, the invention relates to a vector containing a  
20   nucleotide sequence selected from the group consisting of (i) a sequence that provides adenovirus VA RNAs, (ii) an adenovirus E4 ORF6 coding region, (iii) an adenovirus E2a 72 kD coding region (coding for the E2a 72 kD DNA-binding protein), and any combination of  
25   nucleotide sequences (i), (ii) and (iii).

          In another embodiment, the invention relates to nucleic acid molecules which provide accessory functions capable of supporting efficient recombinant AAV (rAAV) virion production in a suitable host cell  
30   and that lack at least one adenoviral late gene region and vectors containing the nucleic acid molecules.

          In another aspect, the invention relates to a vector containing a plurality of nucleotide sequences, including a sequence that provides an  
35   adenovirus VA RNA, a sequence comprising an adenovirus E4 coding region and a sequence comprising an adenovirus E2a coding region.

In yet another aspect of the invention, a vector is provided which contains a sequence that provides an adenovirus VA RNA, a sequence comprising an adenovirus E4 coding region, a sequence comprising an adenovirus E2a coding region, and a sequence comprising an adenovirus E1a and E1b coding region.

In another embodiment of the invention, accessory function systems for rAAV virion production are provided, wherein the systems contain a plurality of accessory function vectors which provide accessory function components suitable for supporting efficient AAV virion production in a suitable host cell.

In yet another embodiment of the invention, methods for producing rAAV virions are provided. The methods generally entail (1) introducing an AAV vector into a suitable host cell; (2) introducing an AAV helper construct into the cell, wherein the helper construct is capable of being expressed in the host cell to complement AAV helper functions missing from the AAV vector; (3) introducing one or more accessory function vectors into the host cell, wherein the one or more accessory function vectors provide accessory functions capable of supporting efficient rAAV virion production in the host cell; and (4) culturing the cell to produce rAAV virions.

In a further embodiment, recombinant AAV virions produced by the methods of the present invention are also provided.

These and other embodiments of the subject invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

#### Brief Description of the Figures

Figure 1 depicts the plasmid construct pladen0 1 which includes VA RNA, E4 (containing the ORF 6) and E2a adenoviral gene regions derived from

adenovirus type-5 which were inserted into a pBSII s/k- vector plasmid.

Figure 2 depicts the plasmid construct pladen0 1 E1 which was formed by inserting a 4,102 bp  
5 *BsrGI-Eco47III* fragment (containing the adenovirus type-5 E1a and E1b coding regions) into the pladen0 1 construct.

Figure 3 depicts the plasmid construct pladen0 5 which includes the VA RNA, E4 ORF 6, and E2a  
10 adenoviral gene regions derived from the adenovirus type-2 genome.

#### Detailed Description of the Invention

The practice of the present invention will  
15 employ, unless otherwise indicated, conventional methods of virology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (Current Edition); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., Current Edition); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., Current Edition); *Transcription and Translation* (B. Hames & S. Higgins, eds., Current Edition); *CRC Handbook of Parvoviruses*, vol. I & II (P. Tijessen, ed.); *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.)  
20  
25

As used in this specification and the  
30 appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

#### A. Definitions

35 In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

"Gene transfer" or "gene delivery" refers to methods or systems for reliably inserting foreign DNA into host cells. Such methods can result in transient expression of non-integrated transferred DNA, extrachromosomal replication and expression of transferred replicons (e.g., episomes), or integration of transferred genetic material into the genomic DNA of host cells. Gene transfer provides a unique approach for the treatment of acquired and inherited diseases. A number of systems have been developed for gene transfer into mammalian cells. See, e.g., U.S. Patent No. 5,399,346.

By "vector" is meant any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

By "adeno-associated virus inverted terminal repeats" or "AAV ITRs" is meant the art-recognized regions found at each end of the AAV genome which function together in *cis* as origins of DNA replication and as packaging signals for the virus. AAV ITRs, together with the AAV *rep* coding region, provide for the efficient excision and rescue from, and integration of a nucleotide sequence interposed between two flanking ITRs into a mammalian cell genome.

The nucleotide sequences of AAV ITR regions are known. See, e.g., Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801; Berns, K.I. "Parvoviridae and their Replication" in *Fundamental Virology*, 2nd Edition, (B.N. Fields and D.M. Knipe, eds.) for the AAV-2 sequence. As used herein, an "AAV ITR" need not have the wild-type nucleotide sequence depicted, but may be altered, e.g., by the insertion, deletion or

substitution of nucleotides. Additionally, the AAV ITR may be derived from any of several AAV serotypes, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, etc. Furthermore, 5' and 3' ITRs which flank a selected nucleotide sequence in an AAV vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the heterologous sequence into the recipient cell genome when AAV Rep gene products are present in the cell.

By "AAV rep coding region" is meant the art-recognized region of the AAV genome which encodes the replication proteins of the virus which are collectively required for replicating the viral genome and for insertion of the viral genome into a host genome during latent infection, or functional homologues thereof such as the human herpesvirus 6 (HHV-6) rep gene which is also known to mediate AAV-2 DNA replication (Thomson et al. (1994) *Virology* 204:304-311). Thus, the rep coding region includes at least the genes encoding for AAV Rep 78 and Rep 68 (the "long forms of Rep"), and Rep 52 and Rep 40 (the "short forms of Rep"), or functional homologues thereof. For a further description of the AAV rep coding region, see, e.g., Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; and Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801. The rep coding region, as used herein, can be derived from any viral serotype, such as the AAV serotypes described above. The region need not include all of the wild-type genes but may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the rep genes present provide for sufficient integration functions when expressed in a suitable recipient cell.



By "AAV cap coding region" is meant the art-recognized region of the AAV genome which encodes the coat proteins of the virus which are collectively required for packaging the viral genome. Thus, the cap coding region includes at least the genes encoding for the coat proteins VP1, VP2 and VP3. For a further description of the cap coding region, see, e.g., Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; and Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801. The AAV cap coding region, as used herein, can be derived from any AAV serotype, as described above. The region need not include all of the wild-type cap genes but may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the genes provide for sufficient packaging functions when present in a host cell along with an AAV vector.

By an "AAV vector" is meant a vector derived from an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, etc. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, preferably the rep and/or cap genes, but retain functional flanking ITR sequences. Functional ITR sequences are necessary for the rescue, replication and packaging of the AAV virion. Thus, an AAV vector is defined herein to include at least those sequences required in cis for replication and packaging (e.g., functional ITRs) of the virus. The ITRs need not be the wild-type nucleotide sequences, and may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the sequences provide for functional rescue, replication and packaging.

"AAV helper functions" refer to AAV-derived coding sequences which can be expressed to provide AAV gene products that, in turn, function in trans for

productive AAV replication. Thus, AAV helper functions include both of the major AAV open reading frames (ORFs), *rep* and *cap*. The Rep expression products have been shown to possess many functions, including, among others: recognition, binding and nicking of the AAV origin of DNA replication; DNA helicase activity; and modulation of transcription from AAV (or other heterologous) promoters. The Cap expression products supply necessary packaging functions. AAV helper functions are used herein to complement AAV functions in trans that are missing from AAV vectors.

The term "AAV helper construct" refers generally to a nucleic acid molecule that includes nucleotide sequences providing AAV functions deleted from an AAV vector which is to be used to produce a transducing vector for delivery of a nucleotide sequence of interest. AAV helper constructs are commonly used to provide transient expression of AAV *rep* and/or *cap* genes to complement missing AAV functions that are necessary for lytic AAV replication; however, helper constructs lack AAV ITRs and can neither replicate nor package themselves. AAV helper constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45 which encode both Rep and Cap expression products. See, e.g., Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McCarty et al. (1991) *J. Virol.* 65:2936-2945. A number of other vectors have been described which encode Rep and/or Cap expression products. See, e.g., U.S. Patent No. 5,139,941.

The term "accessory functions" refers to non-AAV derived viral and/or cellular functions upon which AAV is dependent for its replication. Thus, the term captures proteins and RNAs that are required in

AAV replication, including those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of Cap expression products and AAV capsid assembly.

- 5 Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1) and vaccinia virus.

For example, adenovirus-derived accessory  
10 functions have been widely studied, and a number of adenovirus genes involved in accessory functions have been identified and partially characterized. See, e.g., Carter, B.J. (1990) "Adeno-Associated Virus Helper Functions," in *CRC Handbook of Parvoviruses*,  
15 vol. I (P. Tijssen, ed.), and Muzyczka, N. (1992) *Curr. Topics. Microbiol. and Immun.* 158:97-129. Specifically, early adenoviral gene regions E1a, E2a, E4, VAI RNA and, possibly, E1b are thought to participate in the accessory process. Janik et al.  
20 (1981) *Proc. Natl. Acad. Sci. USA* 78:1925-1929. Herpesvirus-derived accessory functions have been described. See, e.g., Young et al. (1979) *Prog. Med. Virol.* 25:113. Vaccinia virus-derived accessory functions have also been described. See, e.g.,  
25 Carter, B.J. (1990), *supra.*, Schlehofer et al. (1986) *Virology* 152:110-117.

The term "accessory function vector" refers generally to a nucleic acid molecule that includes nucleotide sequences providing accessory functions.

- 30 An accessory function vector can be transfected into a suitable host cell, wherein the vector is then capable of supporting AAV virion production in the host cell. Expressly excluded from the term are infectious viral particles as they exist in nature, such as adenovirus,  
35 herpesvirus or vaccinia virus particles. Thus, accessory function vectors can be in the form of a plasmid, phage, transposon or cosmid.

By "capable of supporting efficient rAAV virion production" is meant the ability of an accessory function vector or system to provide accessory functions that are sufficient to complement rAAV virion production in a particular host cell at a level substantially equivalent to or greater than that which could be obtained upon infection of the host cell with an adenovirus helper virus. Thus, the ability of an accessory function vector or system to support efficient rAAV virion production can be determined by comparing rAAV virion titers obtained using the accessory vector or system with titers obtained using infection with an infectious adenovirus. More particularly, an accessory function vector or system supports efficient rAAV virion production substantially equivalent to, or greater than, that obtained using an infectious adenovirus when the amount of virions obtained from an equivalent number of host cells is not more than about 200 fold less than the amount obtained using adenovirus infection, more preferably not more than about 100 fold less, and most preferably equal to, or greater than, the amount obtained using adenovirus infection.

By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

By "AAV virion" is meant a complete virus particle, such as a wild-type (wt) AAV virus particle (comprising a linear, single-stranded AAV nucleic acid genome associated with an AAV capsid protein coat). In this regard, single-stranded AAV nucleic acid molecules of either complementary sense, e.g., "sense" or "antisense" strands, can be packaged into any one AAV virion and both strands are equally infectious.

A "recombinant AAV virion," or "rAAV virion" is defined herein as an infectious, replication-

defective virus composed of an AAV protein shell, encapsidating a heterologous nucleotide sequence of interest which is flanked on both sides by AAV ITRs. A rAAV virion is produced in a suitable host cell which has had an AAV vector, AAV helper functions and accessory functions introduced therein. In this manner, the host cell is rendered capable of encoding AAV polypeptides that are required for packaging the AAV vector (containing a recombinant nucleotide sequence of interest) into infectious recombinant virion particles for subsequent gene delivery.

The term "transfection" is used to refer to the uptake of foreign DNA by a cell, and a cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, e.g., Graham et al. (1973) *Virology*, 52:456, Sambrook et al. (1989) *Molecular Cloning, a laboratory manual*, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) *Basic Methods in Molecular Biology*, Elsevier, and Chu et al. (1981) *Gene* 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties, such as a nucleotide integration vector and other nucleic acid molecules, into suitable host cells.

The term "host cell" denotes, for example, microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of an AAV helper construct, an AAV vector plasmid, an accessory function vector, or other transfer DNA. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" as used herein generally refers to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA

complement as the original parent, due to natural, accidental, or deliberate mutation.

As used herein, the term "cell line" refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

The term "heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally joined together, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct or a vector is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a nucleic acid construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a cell transformed with a construct which is not normally present in the cell would be considered heterologous for purposes of this invention. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

A "coding sequence" or a sequence which "encodes" a particular protein, is a nucleic acid

sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

A "nucleic acid" sequence refers to a DNA or RNA sequence. The term captures sequences that include any of the known base analogues of DNA and RNA such as, but not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

The term DNA "control sequences" refers collectively to promoter sequences, polyadenylation

signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

10           The term "promoter region" is used herein in its ordinary sense to refer to a nucleotide region comprising a DNA regulatory sequence, wherein the regulatory sequence is derived from a gene which is capable of binding RNA polymerase and initiating transcription of a downstream (3'-direction) coding sequence.

          "Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function.

20   Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

30           By "isolated" when referring to a nucleotide sequence, is meant that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. Thus, an "isolated nucleic acid molecule which encodes a particular polypeptide" refers to a nucleic acid molecule which is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however,



the molecule may include some additional bases or moieties which do not deleteriously affect the basic characteristics of the composition.

For the purpose of describing the relative position of nucleotide sequences in a particular nucleic acid molecule throughout the instant application, such as when a particular nucleotide sequence is described as being situated "upstream," "downstream," "3'," or "5'" relative to another sequence, it is to be understood that it is the position of the sequences in the "sense" or "coding" strand of a DNA molecule that is being referred to as is conventional in the art.

"Homology" refers to the percent of identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions which allow for the formation of stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when at least about 80%, preferably at least about 90%, and most preferably at least about 95% of the nucleotides or amino acids match over a defined length of the molecules, as determined using the methods above.

A "functional homologue," or a "functional equivalent" of a given polypeptide includes molecules derived from the native polypeptide sequence, as well as recombinantly produced or chemically synthesized

polypeptides which function in a manner similar to the reference molecule to achieve a desired result. Thus, a functional homologue of AAV Rep68 or Rep78 encompasses derivatives and analogues of those

5 polypeptides--including any single or multiple amino acid additions, substitutions and/or deletions occurring internally or at the amino or carboxy termini thereof--so long as integration activity remains.

10 A "functional homologue," or a "functional equivalent" of a given adenoviral nucleotide region includes similar regions derived from a heterologous adenovirus serotype, nucleotide regions derived from another virus or from a cellular source, as well as

15 recombinantly produced or chemically synthesized polynucleotides which function in a manner similar to the reference nucleotide region to achieve a desired result. Thus, a functional homologue of an adenoviral VA RNA gene region or an adenoviral E2a gene region

20 encompasses derivatives and analogues of such gene regions--including any single or multiple nucleotide base additions, substitutions and/or deletions occurring within the regions, so long as the homologue retains the ability to provide its inherent accessory

25 function to support AAV virion production at levels detectable above background.

#### B. General Methods

Central to the present invention is the

30 development of accessory function systems which allow for the efficient production of rAAV virions in the absence of infection with a helper virus. Unlike prior production methods, accessory functions are provided by introducing one or more vectors, such as

35 plasmids, which contain genes required for complementing rAAV virion production, into a host cell. In this manner, the present accessory function

systems can support the production of commercially significant levels of rAAV virions without significant levels of contaminating helper virus particles, or other contaminating virus products (e.g., the  
5 adenoviral fiber protein). Efficient production of rAAV virions is achieved when rAAV virion yields are obtained at levels that are not lower than about 200 fold less than levels obtained when using adenovirus type-2 infection to provide the accessory functions.

10 The accessory functions are provided on one or more vectors. The vector(s) will include adenoviral- derived nucleotide sequences necessary for rAAV virion production. As explained further below, the sequences present on the accessory function  
15 construct(s) will be determined by the host cell used and can include E1a, E1b, E2a, E4 and VA RNA regions.

While not being bound by any particular theory, the accessory functions provided by the adenovirus E1b, E2a, and E4 early genes are thought to  
20 be required in AAV DNA replication. The accessory functions provided by the adenovirus E1b, E4 and VA RNA gene regions appear to participate in post-transcriptional or translational events in the AAV life cycle. In regard to the accessory functions  
25 provided by E4, only the E4 34 kD protein encoded by open reading frame 6 (ORF 6) of the E4 coding region is clearly required for AAV replication. The accessory functions provided by the adenovirus gene region E1a are thought to be required as modulators to  
30 activate transcription or expression of the other adenovirus gene regions, including E1b, E2a, E4 and VA RNA.

The accessory function vectors of the invention can alternatively include one or more  
35 polynucleotide homologues which replace the adenoviral gene sequences, so long as each homologue retains the ability to provide the accessory functions of the

replaced adenoviral gene. Thus, homologous nucleotide sequences can be derived from another adenoviral serotype (e.g., adenovirus type-2), from another helper virus moiety (e.g. a herpesvirus or vaccinia virus), or can be derived from any other suitable source.

Further, accessory function vectors constructed according to the invention can be in the form of a plasmid, phage, transposon or cosmid. Alternatively, the vector can be in the form of one or more linearized DNA or RNA fragments which, when associated with the appropriate control elements and enzymes, can be transcribed or expressed in a host cell to provide accessory functions. All of the above-described vectors can be readily introduced into a suitable host cell using transfection techniques that are known in the art. Such transfection methods have been described, including calcium phosphate co-precipitation (Graham et al. (1973) *Virology* 52:456-467), direct micro-injection into cultured cells (Capecchi, M.R. (1980) *Cell* 22:479-488), electroporation (Shigekawa et al. (1988) *BioTechniques* 6:742-751), liposome mediated gene transfer (Mannino et al. (1988) *BioTechniques* 6:682-690), lipid-mediated transfection (Felgner et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-7417), and nucleic acid delivery using high-velocity microprojectiles (Klein et al. (1987) *Nature* 327:70-73).

Accessory function vectors can be engineered using conventional recombinant techniques. Particularly, nucleic acid molecules can be readily assembled in any desired order by inserting one or more accessory function nucleotide sequences into a construct, such as by ligating restriction fragments into a cloning vector using polylinker oligonucleotides or the like. The newly formed nucleic acid molecule can then be excised from the

vector and placed in an appropriate expression construct using restriction enzymes or other techniques that are well known in the art.

More particularly, selected adenoviral genes  
5 or gene regions (e.g., E1a, E1b, E2a, E4 and VA RNA),  
or functional homologues thereof, can be excised from  
a viral genome, or from a vector containing the same,  
and inserted into a suitable vector either  
individually, or linked together, to provide an  
10 accessory function construct using standard ligation  
techniques such as those described in Sambrook et al.,  
*supra*. Referring to Figure 2, one such construct can  
be engineered to include four nucleic acid molecules  
derived from the adenovirus type-5 genome: a VA RNA-  
15 containing region; an E2a- containing region; an E4-  
containing region and an E1a, E1b-containing region.  
Specifically, Figure 2 shows: a 1,724 bp *SalI-HinDIII*  
VA RNA-containing fragment (corresponding to the  
nucleotides spanning positions about 9,831 to about  
20 11,555 of the adenovirus type-2 genome); a 5,962 bp  
*SrfI-BamHI* E2a-containing fragment (corresponding to  
the nucleotides spanning positions about 21,606 to  
about 27,568 of the adenovirus type-2 genome); a 3,669  
bp *HphI-HinDIII* E4-containing fragment (corresponding  
25 to the nucleotides spanning positions about 32,172 to  
about 36,841 of the adenovirus type-2 genome); and a  
4,102 bp *BsrGI-Eco47III* E1a-, E1b-containing fragment  
(corresponding to the nucleotides spanning positions  
about 192 to about 4294 of the adenovirus type-2  
30 genome), wherein the nucleic acid molecules are  
ligated together to provide a complete complement of  
accessory functions in a single accessory function  
construct. Ligations can be accomplished in 20 mM  
Tris-Cl pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 33 ug/ml BSA,  
35 10 mM-50 mM NaCl, and either 40 uM ATP, 0.01-0.02  
(Weiss) units T4 DNA ligase at 0°C (for "sticky end"  
ligation) or 1 mM ATP, 0.3-0.6 (weiss) units T4 DNA

ligase at 14°C (for "blunt end" ligation).

Intermolecular "sticky end" ligations are usually performed at 30-100 µg/ml total DNA concentrations (5-100 nM total end concentration). The assembled

5 molecule can then be readily inserted into an expression vector which is capable of transferring the accessory function construct between cells.

In the alternative, nucleic acid molecules comprising one or more accessory functions can be  
10 synthetically derived, using a combination of solid phase direct oligonucleotide synthesis chemistry and enzymatic ligation methods which are conventional in the art. Synthetic sequences may be constructed having features such as restriction enzyme sites, and  
15 can be prepared in commercially available oligonucleotide synthesis devices such as those devices available from Applied Biosystems, Inc. (Foster City, CA) using the phosphoramidite method. See, e.g., Beaucage et al. (1981) *Tetrahedron Lett.*  
20 22:1859-1862. The nucleotide sequence of the adenovirus type-2 genome is generally known, and is publicly available (e.g., as GeneBank Reference Name: ADRCG, Accession Number: J01917; and as NCBI Identification Number: 209811). The nucleotide  
25 sequence of the adenovirus type-5 genome is believed to be 99% homologous to the adenovirus type-2 genome. Preferred codons for expression of the synthetic molecule in mammalian cells can also be readily synthesized. Complete nucleic acid molecules are then  
30 assembled from overlapping oligonucleotides prepared by the above methods. See, e.g., Edge, *Nature* (1981) 292:756; Nambair et al. *Science* (1984) 223:1299; Jay et al. *J. Biol. Chem.* (1984) 259:6311.

When ad noviral gene regions are used in the  
35 vectors of the invention to provide accessory functions, those regions will be operably linked to control sequences that direct the transcription or

expression thereof. Such control sequences can comprise those adenoviral control sequences normally associated with the gene regions in the wild-type adenoviral genome. Alternatively, heterologous control sequences can be employed where desired. Useful heterologous promoter sequences include those derived from sequences encoding mammalian genes or viral genes. Examples include, but are not limited to, homologous adenoviral promoters, the SV40 early promoter, mouse mammary tumor virus LTR promoter; adenovirus major late promoter (Ad MLP); a herpes simplex virus (HSV) promoter, a cytomegalovirus (CMV) promoter (e.g., the CMV immediate early promoter region), a rous sarcoma virus (RSV) promoter, synthetic promoters, hybrid promoters, and the like. In addition, sequences derived from nonviral genes, such as the murine metallothionein gene, will also find use herein. Such promoter sequences are commercially available from, e.g., Stratagene (San Diego, CA).

Furthermore, the vectors of the present invention can be constructed to also include selectable markers. Suitable markers include genes which confer antibiotic resistance or sensitivity, or impart color, or change the antigenic characteristics when cells which have been transfected with the nucleic acid constructs are grown in an appropriate selective medium. Particular selectable marker genes useful in the practice of the invention include the hygromycin B resistance gene (encoding Aminoglycoside phosphotranferase (APH)) that allows selection in mammalian cells by conferring resistance to G418 (available from Sigma, St. Louis, Mo.). Other suitable markers are known to those of skill in the art.

Accessory function vectors containing a full complement of the adenoviral accessory function genes

or gene regions (e.g., E1a, E1b, E2a, E4, VA RNA, and/or functional homologues thereof) can be used to supply accessory functions to a host cell, including those cells not permissive for helper viruses (e.g.,  
5 not infectable by a helper virus such as an adenovirus or not capable of supporting helper virus replication). In this manner, rAAV virion production can be carried out in a wide range of host cells, including those which were previously refractive to  
10 supporting such production.

In the alternative, accessory function vectors can be constructed to contain less than a full complement of accessory functions. Such vectors can be used in a cell that is already capable of supplying  
15 one or more accessory functions, for example, in a cell that supplies one or more accessory functions either inherently (e.g., where the cell expresses an accessory function homologue) or due to a transformation event. Accessory function vectors  
20 containing less than a full complement of accessory functions can also be used in combination with other ancillary accessory function constructs.

Particularly, suitable host cells can be engineered using ordinary recombinant techniques to  
25 produce cells that provide one or more accessory functions. For example, the human cell line 293 is a human embryonic kidney cell line that has been transformed with adenovirus type-5 DNA fragments (Graham et al. (1977) *J. Gen. Virol.* 36:59), and  
30 expresses the adenoviral E1a and E1b genes (Aiello et al. (1979) *Virology* 94:460). The 293 cell line is readily transfected, and provides a particularly convenient platform in which to produce rAAV virions. Thus, in one particularly preferred embodiment of th  
35 invention, an accessory function vector is provided having only the



adenoviral E2a, E4 and VA RNA gene regions, or functional homologues thereof.

Referring to Figure 1, one such construct can be engineered to include three nucleic acid molecules derived from the adenovirus type-5 genome: a 1,724 bp *SalI-HinDIII* VA RNA-containing fragment (corresponding to the nucleotides spanning positions about 9,831 to about 11,555 of the adenovirus type-2 genome); a 5,962 bp *SrfI-BamHI* E2a-containing fragment (corresponding to the nucleotides spanning positions about 21,606 to about 27,568 of the adenovirus type-2 genome); and a 3,669 bp *HphI-HinDIII* E4-containing fragment (corresponding to the nucleotides spanning positions about 32,172 to about 36,841 of the adenovirus type-2 genome), wherein the nucleic acid molecules are ligated together to provide a truncated complement of accessory functions in a single accessory function construct.

Referring to Figure 3, an alternative construct can be engineered to include three nucleic acid molecules derived from the adenovirus type-2 genome: a 732 bp *EcoRV-SacII* VA RNA-containing fragment (corresponding to the nucleotides spanning positions about 10,423-11,155 of the adenovirus type-2 genome); a 5,962 bp *SrfI-KpnI* E2a-containing fragment (corresponding to the nucleotides spanning positions about 21,606 to about 27,568 of the adenovirus type-2 genome); and a 3,192 bp modified *SrfI-SpeI* E4 ORF6-containing fragment (corresponding to the nucleotides spanning positions about 32,644 to about 34,120 of the adenovirus type-2 genome. The nucleic acid molecules are ligated together to provide an even further truncated complement of accessory functions in a single accessory function construct.

These vectors can be constructed as described above using recombinant and/or synthetic techniques, and can include a variety of ancillary

components such as heterologous promoter regions, selectable markers and the like. Upon transfection into a host 293 cell, the vectors provide accessory functions that are capable of supporting efficient  
5 rAAV virion production.

Once engineered, the accessory function vectors of the present invention can be used in a variety of systems for rAAV virion production. For example, suitable host cells that have been  
10 transfected with one or more accessory function vectors are thereby rendered capable of producing rAAV virions when co-transfected with an AAV vector and an AAV helper construct capable of being expressed in the cell to provide AAV helper functions.

15 The AAV vector, AAV helper construct and the accessory function vector(s) can be introduced into the host cell, either simultaneously or serially, using transfection techniques described above.

AAV vectors used to produce rAAV virions for  
20 delivery of a nucleotide sequence of interest can be constructed to include one or more heterologous nucleotide sequences flanked on both ends (5' and 3') with functional AAV ITRs. In the practice of the invention, an AAV vector generally includes at least  
25 one AAV ITR and an appropriate promoter sequence suitably positioned relative to a heterologous nucleotide sequence, and at least one AAV ITR positioned downstream of the heterologous sequence. The 5' and 3' ITRs need not necessarily be identical  
30 to, or derived from, the same AAV isolate, so long as they function as intended.

Suitable heterologous nucleotide sequences for use in AAV vectors include any functionally relevant nucleotide sequence. Thus, AAV vectors for  
35 use in the practice of the invention can include any desired gene that encodes a protein that is defective or missing from a recipient cell genome or that

encodes a non-native protein having a desired biological or therapeutic effect (e.g., an antiviral function), or the sequence can correspond to a molecule having an antisense or ribozyme function.

5 Suitable genes include, but are not limited to, those used for the treatment of inflammatory diseases, autoimmune, chronic and infectious diseases, including such disorders as AIDS, cancer, neurological diseases, cardiovascular disease, hypercholesterolemia; various  
10 blood disorders including various anemias, thalassemias and hemophilia; genetic defects such as cystic fibrosis, Gaucher's Disease, adenosine deaminase (ADA) deficiency, emphysema, or the like. A number of antisense oligonucleotides (e.g., short  
15 oligonucleotides complementary to sequences around the translational initiation site (AUG codon) of an mRNA) that are useful in antisense therapies for cancer, cardiovascular, and viral diseases have been described in the art. See, e.g., Han et al. (1991) *Proc. Natl.*  
20 *Acad. Sci. USA* 88:4313-4317; Uhlmann et al. (1990) *Chem. Rev.*, 90:543-584; Helene et al. (1990) *Biochim. Biophys. Acta.* 1049:99-125; Agarwal et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:7079-7083; and Heikkila et al. (1987) *Nature* 328:445-449. For a discussion of  
25 suitable ribozymes, see, e.g., Cech et al. (1992) *J. Biol. Chem.* 267:17479-17482 and U.S. Patent No. 5,225,347 to Goldberg et al.

AAV vectors can also include control sequences, such as promoter and polyadenylation sites,  
30 as well as selectable markers or reporter genes, enhancer sequences, and other control elements which allow for the induction of transcription. Such AAV vectors can be constructed using techniques well known in the art. See, e.g., U.S. Patent No. 5,173,414;  
35 International Publication Numbers WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al. (1988) *Molec. Cell.*

- Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines* 90 (Cold Spring Harbor Laboratory Press); Carter, B.J. (1992) *Current Opinion in Biotechnology* 3:533-539; Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801; Shelling and Smith (1994) *Gene Therapy* 1:165-169; and Zhou et al. (1994) *J. Exp. Med.* 179:1867-1875.

In the methods of the invention, AAV helper constructs are used to complement AAV functions deleted from an AAV vector. A number of suitable AAV helper constructs have been described, including, e.g., the plasmids pAAV/Ad and pIM29+45 which encode both *rep* and *cap* expression products (see, e.g., Samulski et al. (1989) *J. Virol.* 63:3822-3828 and McCarty et al. (1991) *J. Virol.* 65:2936-2945). Complementing AAV helper functions in this manner to support rAAV virion production is an art-accepted technique. However, due to homologous recombination events between the AAV ITR sequences present in the AAV vector and the AAV helper function sequences present in the helper construct, such techniques also generate contaminating wild-type AAV virions in the rAAV virion stocks. The presence of wild-type AAV particles in AAV-based vector systems could potentially lead to unintentional spread of recombinant AAV virions, and may interfere with the efficient expression of foreign genes.

### 30 C. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts,

temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Plasmid Construction:

5                   Plasmid JM17 (McGrory et al. (1988) *Virology*  
163:614-617), which comprises a circularized isolate  
of adenovirus type-5 with the plasmid vector pBR322X  
inserted at the unique *Xba*I site at the end of the  
adenovirus *Ela* gene, was used as a source of  
10   adenovirus genes. Neither adenovirus type-5 nor pJM17  
has been completely sequenced. Accordingly, the sizes  
of the adenovirus type-5 fragments described below  
have been approximated. Since adenovirus type-2 has  
been fully sequenced, and adenovirus types 2 and 5 are  
15   thought to be approximately 99% homologous, the  
adenovirus type-5 fragment sizes are approximated  
based upon corresponding adenovirus type-2 fragments.

                  Plasmid pBSII-VA RNAs (ATCC Accession Number  
98233) was constructed as follows. The approximately  
20   5,324 bp *Hin*DIII-*Hin*DIII fragment (containing the  
adenovirus type-5 VA RNA I and II coding regions) was  
obtained from the plasmid pJM17. The 5,324 bp  
fragment was inserted into the plasmid vector pBSII  
s/k- (obtained from Stratagene) at the *Hin*DIII site.  
25   The 5,324 bp fragment corresponds to the nucleotides  
extending from position 6,231 to 11,555 (*Hin*DIII  
sites) of the adenovirus type-2 genome (publicly  
available, e.g., as GeneBank Reference Name: ADRCG,  
Accession Number: J01917; and NCBI Identification  
30   Number: 209811).

                  Plasmid pBSII-E2a+E4 was constructed as  
follows. The approximately 15,667 bp *Bam*HI-*Xba*I  
fragment (containing the adenovirus type-5 E2a, E3 and  
E4 coding regions, adenoviral terminal repeats ligated  
35   head to head, and a portion of the adenovirus type-5  
*Ela* coding region) was obtained from the plasmid  
pJM17. The plasmid vector pBSII s/k- was cut with

*Bam*HI and *Xba*I, and the 15,667 bp fragment was cloned into the subject vector. The 15,667 bp fragment corresponds to the nucleotides extending from position 21,606 (a *Bam*HI site) to 35,937 (the distal end of the 3' terminus), and the nucleotides extending from position 1 (the beginning of the 5' terminus) to 1,336 (an *Xba*I site) of the adenovirus type-2 genome.

Plasmid pBSII-E2a was constructed as follows. The approximately 5,935 bp *Bam*HI-*Eco*RI fragment (containing the adenovirus type-5 E2a coding region) was obtained from the plasmid pJM17. The plasmid vector pBSII s/k- was cut with *Bam*HI and *Eco*RI, and the 5,935 bp fragment was cloned into the subject vector. The 5,935 bp fragment corresponds to the nucleotides extending from position 15,403 (a *Bam*HI site) to 21,338 (an *Eco*RI site) of the adenovirus type-2 genome.

Plasmid pBSII-E4 was constructed as follows. The approximately 5,111 bp *Xho*I-*Xba*I fragment (containing the adenovirus type-5 E4 coding region, adenoviral terminal repeats ligated head to head, and a portion of the adenovirus type-5 E1 coding region) was obtained from pJM17. The plasmid vector pBSII s/k- was cut with *Xho*I and *Xba*I, and the 5,111 bp fragment was cloned into the subject vector. The 5,111 bp fragment corresponds to the nucleotides extending from position 29,788 (an *Xho*I site) to 35,937 (the end of the 3' terminus), and the nucleotides extending from position 1 (the beginning of the 5' terminus) to 1,336 (an *Xba*I site) of the adenovirus type-2 genome.

Plasmid pWadhIacZ was constructed as follows. The plasmid pUC119 (GeneBank Reference Name: U07649, GeneBank Accession Number: U07649) was partially digested with *Afl*III and *Bsp*HI, blunt-end modified with the klenow enzyme, and then ligated to form a circular 1732 bp plasmid containing the

bacterial origin and the *amp* gene only (the polylinker and F1 origin was removed). The blunted and ligated *Afl*III and *Bsp*HI junction forms a unique *Nsp*I site. The 1732 bp plasmid was cut with *Nsp*I, blunt-end modified with T4 polymerase, and a 20 bp *Hin*DIII-*Hin*CII fragment (blunt-end modified with the klenow enzyme) obtained from the pUC119 polylinker was ligated into the blunted *Nsp*I site of the plasmid. The *Hin*DIII site from the blunted polylinker was regenerated, and then positioned adjacent to the bacterial origin of replication. The resulting plasmid was then cut at the unique *Pst*I/*Sse*8387I site, and an *Sse*8387I-*Pvu*II-*Sse*8387I oligonucleotide (5'-GGCAGCTGCCTGCA-3' (SEQ ID No.:\_\_\_\_)) was ligated in. The remaining unique *Bsp*HI site was cut, blunt-end modified with klenow enzyme, and an oligonucleotide containing an *Asc*I linker (5'-GAAGGCGCGCCTTC-3' (SEQ ID No.:\_\_\_\_)) was ligated therein, eliminating the *Bsp*HI site. The resulting plasmid was called pWee.

In order to create the pWadh<sub>lacZ</sub> construct, a CMV<sub>lacZ</sub> expression cassette (comprising a nucleotide sequence flanked 5' and 3' by AAV ITRs, wherein the nucleotide sequence contains the following elements: a CMV promoter, the hGH 1st intron, an adh<sub>lacZ</sub> fragment and an SV40 early polyadenylation site) was inserted into the unique *Pvu*II site of pWee using multiple steps such that the CMV promoter was arranged proximal to the bacterial *amp* gene of pWee.

More particularly, a CMV<sub>lacZ</sub> expression cassette was derived from the plasmid psub201CMV, which was constructed as follows. An oligonucleotide encoding the restriction enzyme sites: *Not*I-*Mlu*I-*Sna*BI-*Age*I-*Bst*BI-*Bss*HII-*Nco*I-*Hpa*I-*Bsp*EI-*Pml*II-*Rsr*II-*Not*I and having the following nucleotide sequence: 5'-GCGGCCGCACGCGTACGTACCGGTTCTGAAGCGCGC ACGGCCGACCATGGTTAACTCCGGACACGTGCGGACCGCGGCCGC-3' (SEQ

ID No.:\_\_\_\_) was synthesized and cloned into the blunt-end modified *KasI-EarI* site (partial) of pUC119 to provide a 2757 bp vector fragment. A 653 bp *SpeI-SacII* fragment containing a nucleotide sequence encoding a CMV immediate early promoter was cloned into the *SnaBI* site of the 2757 bp vector fragment. Further, a 269 bp PCR-produced *BstBI-BstBI* fragment containing a nucleotide sequence encoding the hGH 1st intron which was derived using the following primers: 5'—AAAATTCGAACCTGGGGAGAAACCAGAG—3' (SEQ ID NO.:\_\_\_\_) and 3'—aaaattcgaacaggttaagcgcccctTTG—5' (SEQ ID NO.:\_\_\_\_), was cloned into the *BstBI* site of the 2757 bp vector fragment, and a 135 bp *HpaI-BamHI* (blunt-end modified) fragment containing the SV40 early polyadenylation site from the pCMV- $\beta$  plasmid (obtained from Clontech) was cloned into the *HpaI* site of the subject vector fragment to result in a plasmid called p1.1c. The p1.1c plasmid was then cut with *NotI* to provide a first CMV expression cassette.

The plasmid psub201 (Samulski et al. (1987) *J. Virol* 61:3096-3101) was cut with *XbaI*, blunt-end modified, and *NotI* linkers (5'—TTGCGGCCGCAA—3' (SEQ ID No.\_\_\_\_)) were ligated to the ends to provide a vector fragment containing the bacterial origin of replication and an *amp* gene, wherein the vector fragment is flanked on both sides by *NotI* sites. After being cut with *NotI*, the first CMV expression cassette was cloned into the psub201 vector fragment to create psub201CMV. The ITR-bounded expression cassette from this plasmid was isolated by cutting with *PvuII*, and ligated to pWee after that plasmid was cut with *PvuII* to create pWCMV. pWCMV was then cut with *BssHII* (partial), and a 3246 bp fragment containing the *adh1lacZ* gene (a *SmaI-DraI* nucleotide fragment obtained from the plasmid pCMV- $\beta$ , having *AscI* linkers (5'—GAAGGCGCGCCTTC—3' (SEQ ID No.:\_\_\_\_)) ligated to the ends to provide a 3246 bp fragment) was



ligated into the *Bss*HII site of pWCMV to obtain the pWadh<sub>lac</sub>Z construct.

Plasmid pW1909adh<sub>lac</sub>Z was constructed as follows. A 4723 bp *Spe*I-*Eco*RV fragment containing the AAV *rep* and *cap* encoding region was obtained from the plasmid pGN1909 (ATCC Accession Number 69871). The pGN1909 plasmid is a high efficiency AAV helper plasmid having AAV *rep* and *cap* genes with an AAV p5 promoter region that is arranged in the construct to be downstream from its normal position (in the wild type AAV genome) relative to the *rep* coding region. The 4723 bp fragment was blunt-end modified, and *Asc*I linkers (5'-GAAGGCGCGCCTTC-3' (SEQ ID No.:\_\_\_)) were ligated to the blunted ends. The resultant fragment was then ligated into the unique *Asc*I site of pWadh<sub>lac</sub>Z and oriented such that the AAV coding sequences were arranged proximal to the bacterial origin of replication in the construct.

Plasmid pW620adh<sub>lac</sub>Z was constructed as follows. A 4439 bp *Msc*I fragment containing the AAV *rep* and *cap* encoding region was obtained from the plasmid pSM620 (Samulski et al. (1982) Proc. Natl. Acad. Sci. USA 79:2077-2081. The 4439 bp fragment was blunt-end modified, and *Asc*I linkers (5'-GAAGGCGCGCCTTC-3' (SEQ ID No.:\_\_\_)) were ligated to the blunted ends. The resultant fragment was then ligated into the unique *Asc*I site of pWadh<sub>lac</sub>Z and oriented such that the AAV coding sequences were arranged proximal to the bacterial origin of replication in the construct.

Plasmid pW1909 was constructed as follows. The pW1909adh<sub>Lac</sub>Z plasmid was cut with *Sse*8387, and the 6506 bp *Sse*8387I-*Sse*8387I fragment (containing the ampicillin resistance gene, the *coli* 1 origin of replication, and the AAV helper sequence) was recircularized by intramolecular ligation to provide the pW1909 construct.

The pVlacZ vector plasmid is a modified version of the Sse8387I-Sse8371I fragment obtained from pW1909adh1acZ that has been cloned into the pUC119 plasmid. The Sse8387I-Sse8371I of pVlacZ differs from the Sse8387I-Sse8371I fragment of pW1909 in that all AAV sequences not derived from the AAV inverted terminal repeats (ITRs) have been eliminated. Plasmid pVlacZ was constructed as follows. Synthetic pieces of DNA, formed by combining AAV serotype 2 base pairs 122-145 with a downstream NotI compatible end, were constructed to provide MscI-NotI fragments containing AAV ITR sequences including all of the D loop. The synthetic DNAs were ligated onto both ends of the 4384 bp NotI fragment from the pW1909adh1acZ plasmid. The 4384 bp fragment contains the CMVlacZ sequences. The resulting fragment was then ligated into the 6732 bp MscI fragment of pW1909adh1acZ to provide an assembly construct. The assembly construct was cut with Sse8387I to obtain a 4666 bp Sse8387I-Sse8371I fragment (containing the CMVlacZ sequences) and the 4666 bp fragment was ligated into pUC119 at the Sse8387I site to obtain the pVlacZ vector plasmid.

#### Example 1

##### rAAV Virion Production Using Transfected Adenovirus Genes to Supply Accessory Functions

In order to determine whether adenoviral genes, introduced into a suitable host cell by transfection, are capable of providing accessory functions similar to those provided by an adenoviral infection in the context of AAV replication, the following experiment was conducted.

Cells from the stable human cell line, 293 (readily available through, e.g., the ATCC under Accession Number CRL1573), were plated in eight 10-cm tissue culture dishes at  $1 \times 10^6$  cells/dish to provide 4 duplicate experimental groups. The cells were then

grown at 37 °C to reach 90% confluency over a period of from about 24 to 48 hours prior to transfection. In each group, the plasmid pW1909adh<sub>lacZ</sub> was used as a source of rescuable AAV<sub>lacZ</sub> vector and AAV *rep* and *cap* coding regions.

Transfections were carried out using a modified calcium phosphate method with a total of 20 µg of DNA for a period of 5 hours. More particularly, at 1 to 4 hours prior to transfection, the medium in the tissue culture plates was replaced with fresh DME/F12 culture medium containing 10% FCS, 1% pen/strep and 1% glutamine. A total of 20 µg of DNA, comprising one or more vectors, was added to 1 mL of sterile 300 mM CaCl<sub>2</sub>, which was then added to 1 mL of sterile 2X HBS solution (formed by mixing 280 mM NaCl, 50 mM HEPES buffer, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub> and adjusting the pH to 7.1 with 10 M NaOH) and immediately mixed by gentle inversion. The resultant mixture was pipetted immediately into the 10 cm plates of 90% confluent cells (in 10 mL of the above-described culture medium) and swirled to produce a homogeneous solution. The plates were transferred to a 5% CO<sub>2</sub> incubator and cultured at 37 °C for approximately 5 hours without disturbing. After transfection, the medium was removed from the plates, and the cells washed once with sterile Phosphate buffered saline (PBS).

Adenovirus working stock was prepared by diluting a master stock of adenovirus type-2 to a concentration of 10<sup>6</sup> pfu/mL in DME/F12 plus 10% FCS, 1% pen/strep, 1% glutamine and 25 mM sterile HEPES buffer (pH 7.4).

Cell cultures from the first group were transfected with 10 µg each of the plasmids pW1909adh<sub>lacZ</sub> and pBSII s/k-. After the transfection period, the medium was replaced, 10 mL of medium containing adenovirus type-2 at a multiplicity of

infection (moi) of 1 was added, and the cultures were incubated at 37 °C for approximately 72 hours.

Cell cultures from the second group were transfected with 10 µg each of the plasmids

5 pW1909adh<sub>lacZ</sub>

and pJM17. After the transfection period, the medium was replaced, and the cultures were incubated at 37 °C for approximately 72 hours.

Cell cultures from the third group were  
10 transfected with 10 µg each of pBSII s/k- and pJM17. After the transfection period, the medium was replaced, and the cultures were incubated at 37 °C for approximately 72 hours.

Cell cultures from the fourth group were  
15 transfected with 10 µg each of pW1909adh<sub>lacZ</sub> and pBSII s/k-. After the transfection period, the medium was replaced, and the cultures were incubated at 37 °C for approximately 72 hours.

The cells from each experimental group were  
20 then collected, media was removed by centrifugation (1000 x g for 10 min.), and a 1 mL lysate was produced using 3 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made free of debris by centrifugation (10,000 x g for 10  
25 min). rAAV lacZ virion production was assessed by titering the freeze/thaw extracts on 293 cells, and assaying for lacZ.

Specifically, 293 cells were plated in 12 well plates (at  $1 \times 10^5$  cells per well) and inoculated  
30 with a range of volumes (10-0.01 µL) of the above-described freeze/thaw lysates and incubated for 24 hours at 37 °C. The cells were then fixed and stained by removal of the medium, incubation of the cells for 5 minutes in PBS containing 2% formaldehyde and 0.2%  
35 glutaraldehyde, washing once with PBS, and then incubating the cells over-night in PBS containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2

mM magnesium chloride, and 1 mg/ml X-Gal (Sanes et al. (1986) *EMBO* 5:3133-3142). The rAAV virion titer was then calculated by quantifying the number of blue cells using light microscopy.

5                   Contaminating infectious adenovirus  
production was assayed as follows. Samples from the  
cell lysates were added to 50% confluent 293 cells  
(cultured in 12 well dishes at  $1 \times 10^5$  cells/well),  
and the cultures were passaged for 30 days (e.g., the  
10   cultures were split 1 to 5, every 3 days) or until the  
culture exhibited 100% CPE due to adenovirus  
infection. Cultures were examined daily for CPE, and  
the day upon which each experimental culture showed  
100% CPE was noted. Reference 293 cell cultures  
15   infected with a range of known amounts of adenovirus  
type-2 (from 0 to  $1 \times 10^7$  pfu/culture) were also  
prepared and treated in the same manner. A standard  
curve was then prepared from the data obtained from  
the reference cultures, where the adenovirus pfu  
20   number was plotted against the day of 100% CPE. The  
titer of infectious adenovirus type-2 in each  
experimental culture was then readily obtained as  
determined from the standard curve. The limit of  
detection in the assay was 100 pfu/mL.  
25                   The results of the experiment are depicted  
below in Table 1.

30

35

Table 1				
Group	Transfected Plasmids	ad-2 Infection	rAAV Titer†	Ad novirus Titer
1	pW1909lacZ/pBS	yes	$1 \times 10^{10}$	$\geq 10^9$ pfu/mL
1	pW1909lacZ/pBS	yes	$1 \times 10^{10}$	$\geq 10^9$ pfu/mL
2	pW1909lacZ/pJM17	no	$1 \times 10^9$	none detected
2	pW1909lacZ/pJM17	no	$2 \times 10^9$	none detected
3	pBS/pJM17	no	0	$10^4$ pfu/mL
3	pBS/pJM17	no	0	$10^4$ pfu/mL
4	pW1909lacZ/pBS	no	0	not tested
4	pW1909lacZ/pBS	no	0	not tested

† As determined by the lacZ assay.

As can be seen by the results in Table 1, adenoviral genes introduced into a host cell by transfection and expressed in the absence of adenoviral infection can provide accessory functions at a level that is approximately 20% (e.g., 5 fold less) as effective as the level of accessory functions provided by an adenovirus infection.

### Example 2

#### Identification of Adenoviral Gene

#### Regions Responsible for Accessory Functions

In order to determine which adenoviral genes or gene regions are necessary and sufficient in the provision of accessory functions, the following experiment was conducted.

293 cells were plated in twelve 10-cm tissue culture dishes at  $1 \times 10^6$  cells/dish to provide 6 duplicate experimental groups. The cells were then grown at 37 °C to reach approximately 90% confluency over a period of from about 24 to 48 hours prior to transfections. In each group, the plasmid pW1909adh1lacZ was used as a source of rescuable AAVlacZ vector and AAV rep and cap coding regions.

Transfections were carried out as described above in Example 1. Adenovirus working stock was also prepared as previously described.

Each of the 293<sup>+</sup> cell cultures were  
5 transfected with the plasmid pW1909adh<sub>lacZ</sub> and either the plasmid pBSII s/k<sup>-</sup> (as a control), or various combinations of isolated adenoviral genes.

More particularly, cells in the first  
experimental group were co-transfected with 5  $\mu$ g of  
10 the plasmid pW1909adh<sub>lacZ</sub> (to provide AAV help functions) and 15  $\mu$ g of the plasmid pBSII s/k<sup>-</sup>. After the transfection period, the medium was replaced, and the cells were infected using 10 mL medium containing adenovirus type-2 (moi=1). The cultures were then  
15 incubated at 37 °C for approximately 72 hours.

Cells in the second experimental group were co-transfected with 5  $\mu$ g of the plasmid pW1909adh<sub>lacZ</sub> (to provide AAV help functions), and 15  $\mu$ g of the plasmid pJM17. After the transfection, the medium was  
20 replaced and the cultures were incubated at 37 °C for approximately 72 hours.

Cells in the third experimental group were co-transfected with 5  $\mu$ g of the plasmid pW1909adh<sub>lacZ</sub> (to provide AAV help functions), 10  $\mu$ g of the plasmid  
25 pBSII-E2a+E4, and 5  $\mu$ g of the plasmid pBSII s/k<sup>-</sup>. After the transfection, the medium was replaced, and the cultures were incubated at 37 °C for approximately 72 hours.

Cells in the fourth experimental group were  
30 co-transfected with 5  $\mu$ g of the plasmid pW1909adh<sub>lacZ</sub> (to provide AAV help functions), 10  $\mu$ g of the plasmid pBSII-E2a+E4, and 5  $\mu$ g of the plasmid pBSII-VA RNAs. After the transfection, the medium was replaced, and the cultures were incubated at 37 °C for approximately  
35 72 hours.

Cells in the fifth experimental group were co-transfected with 5  $\mu$ g of the plasmid pW1909adh<sub>lacZ</sub>

(to provide AAV help functions), 5  $\mu$ g of the plasmid pBSII-E2a, 5  $\mu$ g of the plasmid pBSII-E4, and 5  $\mu$ g of the plasmid pBSII s/k-. After the transfection, the medium was replaced, and the cultures were incubated  
5 at 37 °C for approximately 72 hours.

Cells in the sixth experimental group were co-transfected with 5  $\mu$ g of the plasmid pW1909adh<sub>lacZ</sub> (to provide AAV help functions), 5  $\mu$ g of the plasmid pBSII-E2a, 5  $\mu$ g of the plasmid pBSII-E4, and 5  $\mu$ g of  
10 the plasmid pBSII-VA RNAs. After the transfection, the medium was replaced, and the cultures were incubated at 37 °C for approximately 72 hours.

The cells from each experimental group were then collected, media was removed by centrifugation  
15 (1000 x g for 10 min.), and a 1 mL lysate was produced using 3 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made free of debris by centrifugation (10,000 x g for 10 min). rAAV lacZ virion production was then assessed  
20 using the techniques described in Example 1, and the amount of rAAV genomes was quantified by the following assay method.

50 $\lambda$  of the lysate from each experimental group was added to a 100 $\lambda$  aliquot of DMEM medium  
25 (available from Sigma, St. Louis, MO) containing 50 U/mL DNase I to form assay samples. The samples were incubated at 37 °C for approximately 1 hour, after which 100 $\lambda$  of proteinase K (1 mg/mL) in a 2X proteinase K buffer (20 mM Tris Cl, 20 mM EDTA, 1%  
30 SDS, pH adjusted to 8.0) was added to each sample which were then incubated at 37 °C for another hour. DNA from the samples was phenol/chloroform extracted, precipitated in EtOH and then collected by centrifugation at 5 °C for 15 minutes. The DNA  
35 pellets were then redissolved in 200 $\lambda$  TE to provide DNA samples. Dot blot assays were then conducted as follows. Zeta Probe® membrane (Bio Rad, Richmond, CA)



was cut to size and assembled into a dot blot apparatus. The DNA samples were denatured using 200 $\lambda$  of a 2X alkaline solution (0.8 M NaOH, 20 mM EDTA), and, after 5 minutes, the membranes were rinsed in a 2X SSC solution for 1 minute, dried on filter paper, then baked under vacuum at 80 °C for approximately 30 minutes. Hybridizations were carried out at 65 °C for 30 minutes in hybridization buffer (1 mM EDTA, 40 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2), 7% SDS). The filters were then washed and autoradiographed for approximately 20 hours, radioactivity was determined using scintillation counting.

The results from the experiment are depicted below in Table 2.

Table 2				
Group	Transfected Plasmids	ad-2 Infection	rAAV Titer†	Genomes/mL
1	pW1909lacZ	yes	2 x 10 <sup>9</sup>	3 x 10 <sup>11</sup>
2	pW1909lacZ, pJM17	no	2 x 10 <sup>9</sup>	4 x 10 <sup>11</sup>
3	pW1909lacZ, pBSII-E2a+E4	no	4 x 10 <sup>8</sup>	1 x 10 <sup>11</sup>
4	pW1909lacZ, pBSII-E2a+E4, pBSII-VA RNAs	no	3 x 10 <sup>9</sup>	5 x 10 <sup>11</sup>
5	pW1909lacZ, pBSII-E2a, pBSII-E4	no	2 x 10 <sup>8</sup>	1 x 10 <sup>11</sup>
6	pW1909lacZ, pBSII-E2a, pBSII-E4, pBSII-VA RNAs	no	2 x 10 <sup>9</sup>	4 x 10 <sup>11</sup>

† As determined by the lacZ assay.

As can be seen by the results depicted in Table 2, isolated adenoviral gene regions can be successfully transfected into host cells to provide accessory functions that are necessary and sufficient for rAAV virion replication. Further, the results obtained with groups 3 and 5 indicate that the adenoviral VA RNA region is not essential for the

replication of rAAV virions; however, the region is needed to obtain rAAV titers comparable to those obtained using adenoviral infection.

5

### Example 3

#### Correlation of Adenoviral VA RNA Dosage to rAAV Virion Production

In order to investigate whether a correlation exists between the amount of transfected adenoviral VA RNA gene region supplied to a host cell, and the level of accessory functions provided to complement rAAV replication in the host cell, the following experiment was carried out.

293 cells were plated in 10-cm tissue culture dishes at  $1 \times 10^6$  cells/dish, and were cultured at 37 °C to reach approximately 90% confluency over a period of from about 24 to 48 hours prior to transfections. Transfections were carried out as described above in Example 1.

Specifically, 293 cells were transfected with 5 µg of the plasmid pW1909adh<sub>lacZ</sub>, 10 µg of the plasmid pBSII-E2a+E4 and from 0 to 25 µg of the plasmid pBSII-VA RNAs to vary the molar ratio of the VA RNA bearing plasmid (relative to the other plasmids) over the range of 0 to 5. After the transfection period, the medium was exchanged, and the cells were incubated at 37 °C for approximately 72 hours.

Cells from each experimental group were then collected, media was removed by centrifugation (1000 x g for 10 min.), and a 1 mL lysate was produced using 3 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made free of debris by centrifugation (10,000 x g for 10 min). AAV lacZ vector production was then assessed using the techniques described in Example 1. The

results from the experiment are depicted below in Table 3.

5	Table 3		
	Amount of pBSII-VA RNAs Transfected ( $\mu$ g)	Molar Ratio of pBSII-VA RNAs/other plasmids	rAAV Titer†
	0	0	$3 \times 10^8$
	1	0.2	$6 \times 10^9$
10	5	1	$1 \times 10^{10}$
	10	2	$7 \times 10^9$
	25	5	$5 \times 10^9$

† As determined by the lacZ assay.

As can be seen from Table 3, although adenoviral VA RNAs are needed to obtain rAAV virion production at levels substantially equivalent to those obtained with adenoviral infection, variations in the ratio of VA RNA/other adenoviral gene regions over the range investigated does not significantly effect rAAV virion production.

#### Example 4

##### Demonstration of the Requirement for Adenoviral E2a,

##### E4 and VA RNA Gene Regions in Accessory Functions

In order to establish the relative contributions of the adenoviral E2a, E4 and VA RNA gene regions in the provision of accessory functions for rAAV virion production, the following experiment was carried out.

293 cells were plated in 10-cm tissue culture dishes at  $1 \times 10^6$  cells/dish, and were cultured at 37 °C to reach approximately 90% confluency over a period of from about 24 to 48 hours prior to co-transfections. All transfections were carried out as described above in Example 1.

The 293 cell cultures were co-transfected with 5  $\mu$ g of the plasmid pW1909adh<sub>lacZ</sub>, and 15 $\mu$ g (total) of either all, or paired combinations of the following plasmids: pBSII-E2a; pBSII-E4; and pBSII-VA RNAs to provide cultures in which each of the adenoviral gene regions encoding E2a, E4 and VA RNA were eliminated from a co-transfection. After the transfection period, the media was replaced, and the cells were incubated at 37 °C for approximately 72 hours.

The cells from each co-transfection group were then collected, media was removed by centrifugation (1000 x g for 10 min.), and a 1 mL lysate was produced using 3 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made free of debris by centrifugation (10,000 x g for 10 min). rAAV lacZ virion production was then assessed using the techniques described in Example 1. The results from the experiment are depicted below in Table 4.

Table 4			
Co-transfection with Helper Plasmids Encoding			rAAV Titer†
E2a	E4	VA RNAs	
+	+	+	5 x 10 <sup>9</sup>
-	+	+	5 x 10 <sup>5</sup>
+	-	+	6 x 10 <sup>7</sup>
+	+	-	7 x 10 <sup>8</sup>
-	-	-	0

† As determined by the lacZ assay.

As can be seen by the results depicted in Table 4, each of the adenoviral gene regions E2a, E4 and VA RNA are not absolutely essential to provide the accessory functions needed to support rAAV virion

production; however, all of the gene regions are needed to produce levels of rAAV virions comparable to those obtained with adenoviral infection. More particularly, omission of the VA RNA containing plasmid from the co-transfection resulted in a 7 fold drop in rAAV virion production ( $7 \times 10^8$  functional units per 10 cm dish). rAAV virion production was even more severely affected by omission of the E4- and E2a-containing plasmids from the co-transfection. Omission of the E4 construct resulted in an 83 fold drop in production ( $6 \times 10^7$  functional units per 10 cm dish), and omission of the E2 construct resulted in a 10,000 fold drop in rAAV virion production ( $5 \times 10^5$  functional units per 10 cm dish).

#### Example 5

##### Comparison of rAAV Virion Production Using pW1909adh1acZ or pW620adh1acZ Based AAV Help

In order to compare the efficiency of rAAV virion production in a host cell using non-viral accessory function systems with AAV helper constructs containing either wild-type or modified AAV help functions (AAV rep and cap coding regions), the following experiment was carried out.

293 cells were plated in 10-cm tissue culture dishes at  $1 \times 10^6$  cells/dish, and were cultured at 37 °C to reach approximately 90% confluency over a period of from about 24 to 48 hours prior to co-transfections. All transfections were carried out as described above in Example 1.

A first set of 293 cell cultures was co-transfected with 5 µg of the plasmid pW620adh1acZ (containing wild-type AAV help functions), 10 µg of the plasmid pBSII-E2a+E4 and 5 µg of the plasmid pBSII-VA RNAs. A second set of 293 cultures was co-transfected with 5 µg of the plasmid pW1909adh1acZ (containing modified AAV help functions), 10 µg of the

plasmid pBSII-E2a+E4 and 5  $\mu$ g of the plasmid pBSII-VA RNAs. After the transfections, the media was replaced, and the cultures were incubated at 37 °C for approximately 72 hours.

5                Cells from each co-transfection group were then collected, media was removed by centrifugation (1000 x g for 10 min.), and a 1 mL lysate was produced using 3 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made  
10 free of debris by centrifugation (10,000 x g for 10 min). rAAV lacZ virion production was then assessed using the techniques described in Example 1. The results from the experiment are depicted below in Table 5.

15

Table 5	
Vector/Helper Construct	rAAV Titer†
pW1909adh1lacZ	5 x 10 <sup>9</sup>
20      pW620adh1lacZ	4 x 10 <sup>9</sup>

† As determined by the lacZ assay.

As can be seen from the results depicted in Table 5, both the wild-type and modified forms of AAV  
25 help supported rAAV virion production at approximately the same level.

#### Example 6

##### Comparison of rAAV Virion Production

##### Efficiency of Plasmid-Based Accessory Functions

30                In order to compare the efficiency of rAAV virion production in a host cell using plasmid-based isolated accessory functions, combinations thereof, and a single construct containing adenovirus VA RNA,  
35 E4 and E2a gene regions (the pladen 1 plasmid, described below) with rAAV virion production obtained

using adenovirus type-2 (ad-2) infection, the following experiment was carried out.

1. Construction of pladeno 1 and pladeno 1 E1:

5           The plasmid pladeno 1, containing adenovirus  
VA RNA, E4 and E2a gene regions, was assembled by  
cloning adenovirus type-5 genes into a custom  
polylinker that was inserted between the PvuII sites  
of pBSII s/k-. A map of the pladeno 1 construct is  
10 depicted in Figure 1. More particularly, a double  
stranded oligonucleotide polylinker encoding the  
restriction enzyme sites *SalI*-*XbaI*-*EcoRV*-*SrfI*-*BamHI*  
(5'-GTCGACAAATCTAGATATCGCCCGGGCGGATCC-3' (SEQ ID  
No.: \_\_\_\_)) was ligated to the 2513 bp PvuII vector  
15 fragment of pBSII s/k- to provide an assembly plasmid.  
The following fragments containing adenovirus type-5  
genes or gene regions were then obtained from the  
pJM17 plasmid: the 1,724 bp *SalI*-*HinDIII* VA RNA-  
containing fragment (corresponding to the nucleotides  
20 spanning positions about 9,831 to about 11,555 of the  
adenovirus type-2 genome); the 5,962 bp *SrfI*-*BamHI*  
E2a-containing fragment (corresponding to the  
nucleotides spanning positions about 21,606 to about  
27,568 of the adenovirus type-2 genome); and the 3,669  
25 bp *HphI*-*HinDIII* E4-containing fragment (corresponding  
to the nucleotides spanning positions about 32,172 to  
about 36,841 of the adenovirus type-2 genome). An  
*XbaI* site was added to the *HphI* end of the E4-  
containing fragment by cloning the 3,669 bp *HphI*-  
30 *HinDIII* fragment into the *HpaI* site of cloning vector,  
and then excising the fragment with *XbaI* and *HinDIII*  
(partial digestion). The 5,962 E2a-containing  
fragment was cloned between the *SrfI* and *BamHI* sites  
of the assembly plasmid, and the 1,724 bp VA RNA-  
35 containing fragment and the modified 3,669 bp E4-  
containing fragments were joined by their common  
*HinDIII* ends and ligated between the *SalI* and *XbaI*

sites of the assembly plasmid to obtain the pladenos 1 construct.

Referring now to Figure 2, the pladenos 1 E1 plasmid was assembled as follows. The 4,102 bp *BsrGI*-  
5 *Eco47III* fragment (containing the adenovirus type-5 E1a and E1b coding regions) was obtained from the pJM17 plasmid. The subject fragment corresponds to the nucleotides spanning positions about 192 to about 4,294 of the adenovirus type-2 genome. The 4,102 bp  
10 fragment was blunt-end modified, and then inserted into the *HpaI* site in the VA RNA fragment of the pladenos 1 plasmid to obtain the pladenos 1 E1 plasmid.

## 2. rAAV Virion Production Assay:

15 293 cells were plated in 10-cm tissue culture dishes at  $1 \times 10^6$  cells/dish, and were cultured at 37 °C to reach approximately 90% confluency over a period of from about 24 to 48 hours prior to co-transfections. All transfections were  
20 carried out as described above in Example 1.

All of the 293 cell cultures were transfected with 5 µg of the plasmid pW1909adh<sub>lacZ</sub>. Experimental groups of the cultures were also co-transfected with various combinations of 5 µg each of  
25 the accessory function containing plasmids or control plasmid (pBSII s/k-). After the transfections, the media was replaced, and the cultures were incubated at 37 °C for approximately 72 hours. As a comparison, 10 mL of medium containing adenovirus type-2 (moi=1) was  
30 added to 293 cells that had been transfected with 5 µg of the plasmid pW1909adh<sub>lacZ</sub>, and incubated at 37 °C for approximately 72 hours.

Cells from each experimental group were then collected, media was removed by centrifugation (1000 x  
35 g for 10 min.), and a 1 mL lysate was produced using 3 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made



free of debris by centrifugation (10,000 x g for 10 min). rAAV lacZ virion production was then assessed using the techniques described in Example 1. The results from the experiment are depicted below in

5 Table 6.

Table 6	
Transfected Plasmids	AAV Titer†
ad-2 (moi=1)	$5 \times 10^9$
10 pladeno 1	$3 \times 10^9$
pBSII-E2a, pBSII-E4, pBSII-VA RNAs	$1 \times 10^9$
pBSII-E2a+E4, pBSII-VA RNAs	$5 \times 10^8$
pladeno 1, pBSII-E2a	$8 \times 10^9$
15 pladeno 1, pBSII-E4	$2 \times 10^9$
pladeno 1, pBSII-VA RNAs	$2 \times 10^9$
pladeno 1, pBSII-E2a+E4	$1 \times 10^9$
pBSII-E2a	$2 \times 10^7$
pBSII-E4	$<10^4$
20 pBSII-VA RNAs	$3 \times 10^4$
pBSII s/k-	$<10^4$

† As determined by the lacZ assay.

25 As can be seen by the results in Table 6, the pladeno 1 construct is capable of supporting efficient rAAV virion production (at substantially the same level as that obtained using adenovirus type-2 infection). The combination of accessory function constructs pBSII-E2a, pBSII-E4 and pBSII-VA RNAs was  
30 also able to support efficient rAAV virion production at levels substantially equivalent to ad-2 infection. The combination of accessory function constructs pBSII-E2a+E4 and pBSII-VA RNAs was able to support  
35 rAAV production (10 fold less than ad-2 infection levels); and the E2a containing construct (pBSII-E2a)

supported rAAV production at a level approximately 200 fold less than that obtained using ad-2 infection.

#### Example 7

5                    Comparison of Large Scale rAAV Virion  
                    Production Obtained Using  
                    Adenoviral-Based or Plasmid-Based Accessory Functions

                    In order to compare large scale preparations  
of rAAV virions produced using either adenovirus type-  
10    2 (ad-2) based, or pladen0 1 based accessory  
functions, the following experiment was carried out.

                    Approximately  $10^9$  293 cells were transfected  
with an AAV vector containing the human erythropoietin  
gene using the transfection method described in  
15    Example 1. One preparation was co-transfected with  
the pladen0 1 construct, the other preparation was  
infected with adenovirus type-2 as a source of  
accessory functions.

                    After a suitable incubation period, cells  
20    from each experimental group were then collected,  
growth media was removed by centrifugation (1000 x g  
for 10 min.), and a lysate was produced using 3  
freeze/thaw cycles (alternating between dry  
ice-ethanol and 37°C baths). The lysates were made  
25    free of debris by centrifugation (10,000 x g for 10  
min) to obtain a crude lysate. In order to obtain a  
purified sample, the crude lysates were subjected to  
density gradient centrifugation.

                    The amount of rAAV genomes produced by each  
30    preparation was quantified by the dot blot assay  
described in Example 2. The results of the experiment  
are depicted below in Table 7.

Table 7				
	Ad-2		pladeno 1	
	genomes	% recovery	genomes	% recovery
crude lysate	$6.5 \times 10^{13}$	100	$1.6 \times 10^{14}$	100
after purification	$5.5 \times 10^{13}$	85	$8 \times 10^{13}$	50

As can be seen from the results in Table 7, the preparation using the pladeno 1-based accessory functions provided a rAAV virion yield that was 2.4 fold greater than that obtained from the preparation using the adenovirus type-2 based accessory functions.

#### Example 8

#### Determination of the Relative Contributions of Individual Adenoviral Accessory Functions in rAAV Virion Production

In order to determine the relative contributions of the individual adenoviral accessory functions in rAAV virion production, the following experiment was carried out. Individual adenoviral accessory functions, either alone, or in combinations, were used to support rAAV virion production in host cells. In addition, the effect of substituting CMV-driven E2a or E4 ORF6 constructs (the p3.3cE2A and p3.3cE4ORF6 constructs, described below), for constructs containing the entire E2a and E4 ORF6 regions and driven by homologous promoters (the pBSII-E2a and pBSII-E4 constructs) was assessed.

#### 1. Construction of p3.3cE2A and p3.3cE4ORF6:

The structural genes encoding the adenovirus type-5 E2a 72 kD DNA-binding protein, and the adenovirus type-5 E4 open reading frame 6 (ORF6) protein, were each cloned into a CMV driven expression

construct, p3.3c, to provide the p3.3cE2A and p3.3E4ORF6 plasmid constructs, respectively.

Plasmid p3.3c was constructed as follows. The 2732 bp *NotI* fragment from p1.1c, which contains  
5 pUC119 vector sequences, was ligated to a synthetic DNA fragment containing the following restriction sites: *NotI*; *MluI*; *Ecl136II*; *SacII*; *BstBI*; *BssHII*; *SrfI*; *BssHII*; *BglII*; *SnaBI*; *BstEII*; *PmlI*; *RsrII*; and *NotI*. The sequence of the synthetic DNA fragment is:  
10 5'-GCGGCCGCACGCGTGAGCTCCGCGGTTCGAAGCGCGCAAAGCCCCGGGCAAA  
GCGCGCAGATCTACGTAGGTAACCACGTGCGGACCGGCGGCCGC-3' (SEQ ID No.:\_\_\_\_). A 653 bp *SpeI*-*SacII* fragment including the cytomegalovirus immediate early (CMV IE) promoter; a 269 bp PCR-produced *BstBI*-*BstBI* fragment encoding  
15 the hGH 1st intron that was obtained using the following primers 5'-AAAATTCGAACAGGTAAGCGCCCCTTTG-3' (SEQ ID No.:\_\_\_\_) and 3'-AAAATTCGAATCCTGGGGAGAAAC CAGAG-5' (SEQ ID No.:\_\_\_\_); and a 213 bp *BamHI*-*BamHI* (blunted) fragment containing the SV40 late  
20 polyadenylation site from pCMV- $\beta$  (obtained from Clonetech), were cloned into the *Ecl136II*, *BstBI* and *SnaBI* sites of the synthetic linker, respectively, to result in the p3.3c expression construct.

Plasmid p3.3cE4ORF6 (ATCC Accession Number  
25 98234) was prepared as follows. The 1024 bp *BglII*-*SmaI* fragment from pBSII-E4, containing sequences encoding the adenovirus type-5 E4 ORF6, was obtained and blunt end-modified. This fragment corresponds to position 33,309 (*SmaI* site) through position 34,115  
30 (*BglII* site) of the adenovirus type-2 genome. The modified fragment was then cloned into the *SrfI* site of p3.3c to provide the p3.3cE4ORF6 plasmid.

Plasmid p3.3cE2A (ATCC Accession Number 98235) was prepared as follows. The 2467 bp  
35 *MscI*(partial)-*BamHI* fragment from pBSII-E2a was obtained. This fragment contains adenovirus type-5 E2a coding sequences and corresponds to positions

24,073 (*MscI* site) through 21,606 (*BamHI* site) of the adenovirus type-2 genome. The 2467 bp fragment was subcloned between the *MscI* and *BamHI* sites of pCITE2A (obtained from Novagene) to provide the pCITE2AE2A construct. The 1636 bp *NcoI*(partial)-*BsrGI* fragment was then excised from pCITE2AE2A. This fragment contains sequences encoding the E2a 72 kD protein, and corresponds to positions 24,076 (*MscI/NcoI* site) through 22,440 (*BsrGI* site) of the adenovirus type-2 genome. The 1636 bp fragment was blunt end-modified, and cloned into p3.3c to provide the p3.3cE2A plasmid.

## 2. rAAV Virion Production Assay:

293 cells were plated in 10-cm tissue culture dishes at  $2 \times 10^6$  cells/dish, and were cultured at 37 °C to reach approximately 90% confluency over a period of about 48 hours prior to co-transfections. All transfections were carried out using the  $\text{CaPO}_4$  method with the following combinations of DNAs. All of the 293 cell cultures were transfected with 5 µg of the plasmid pW620adh<sub>lacZ</sub>. 5 µg of various accessory function constructs (as shown in Table 8 below) were used to provide a total of 20 µg DNA per dish. For samples receiving less than 4 constructs, pBSII DNA was used to bring the total amount of transfected DNA to 20 µg. After the transfections, the media was replaced, and cultures using adenovirus for accessory functions received adenovirus type-2 at a MOI of 5. All cultures were then incubated at 37 °C for approximately 72 hours prior to harvest.

Cells from each dish were then collected, media was removed by centrifugation (1000 x g for 10 min.), and a 1 mL lysate was produced using 3 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made free of debris by centrifugation (10,000 x g for 10

min). rAAV lacZ virion production was then assessed using the techniques described in Example 1. The lacZ titering was done in the presence of adenovirus. All samples were assayed in duplicate. The results from the experiment are depicted below in Table 8.

Table 8	
Adenoviral Accessory Functions	AAV Titer†
ad-2 (moi=5)	$4 \times 10^8$
pBSII-E2a, pBSII-E4, pBSII-VA RNAs	$6 \times 10^8$
pladeno 1	$8 \times 10^8$
pBSII-E2a	$8 \times 10^6$
pBSII-E4	$<10^4$
pBSII-VA RNAs	$5 \times 10^4$
pBSII-E4, pBSII-VA RNAs	$1 \times 10^5$
pBSII-E2a, pBSII-VA RNAs	$6 \times 10^7$
pBSII-E2a, pBSII-E4	$1 \times 10^8$
p3.3cE2A, pBSII-E4, pBSII-VA RNAs	$4 \times 10^8$
pBSII-E2a, p3.3cE4ORF6, pBSII-VA RNAs	$3 \times 10^8$
p3.3cE2A, p3.3cE4ORF6, pBSII-VA RNAs	$4 \times 10^8$

† As determined by the lacZ assay.

As can be seen by the results depicted in Table 8, the adenoviral E2a, E4 and VA RNA regions are all necessary to provide efficient rAAV virion production (at levels substantially equivalent to those obtained using adenovirus infection to provide the accessory functions). However, no single one of those regions is absolutely required for rAAV virion production.

The E2a and E4 regions that are subcloned into the pBSII-E2a and pBSII-E4 constructs contain several open reading frames (ORFs) in addition to the

ORFs for the 72 kD E2a DNA binding protein and the E4 ORF6 protein. By substituting the CMV-driven p3.3cE2A and p3.3cE4ORF6 constructs for the pBSII-E2a and pBSII-E4 constructs in the above-described study, it  
5 has now been established that the E2a 72 kD DNA-binding protein and the E4 ORF6 protein are capable of providing full E2a or E4 accessory function in the absence of all other open reading frames from their respective coding regions.

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#### Example 9

#### Reconstruction of the pladeno 1 Plasmid and Comparison of rAAV Virion Production Efficiency

The pladeno 1 plasmid was reconstructed  
15 using purified adenovirus type-2 DNA as a source of the adenoviral genes in place of the pJM17-derived adenovirus genes that were used in the construction of pladeno 1. The reconstructed plasmid, termed pladeno 5, is described in detail below. This reconstruction  
20 was carried out to reduce the overall size of the plasmid. Furthermore, the reconstructed plasmid (pladeno 5) does not encode the adenovirus protease, whereas pladeno 1 does.

#### 25 1. Construction of pladeno 5:

The pladeno 5 plasmid was constructed as follows. DNA fragments encoding the E2a, E4 and VA RNA regions isolated from purified adenovirus type-2 DNA (obtained from Gibco/BRL) were ligated into a  
30 plasmid called pAmpscript. The pAmpscript plasmid was assembled as follows: oligonucleotide-directed mutagenesis was used to eliminate a 623 bp region including the polylinker and alpha complementation expression cassette from pBSII s/k+ (obtained from  
35 Stratagene), and to replace it with an EcoRV site. The sequence of the mutagenic oligo used on the oligonucleotide-directed mutagenesis was

5'—CCGCTACAGGGCGCGATATCAGCTCACTCAA—3' (SEQ ID No.:\_\_\_\_). A polylinker (containing the following restriction sites: *Bam*HI; *Kpn*I; *Srf*I; *Xba*I; *Cla*I; *Bst*1107I; *Sal*I; *Pme*I; and *Nde*I) was synthesized and  
5 inserted into the *Eco*RV site created above such that the *Bam*HI side of the linker was proximal to the *f1* origin in the modified plasmid to provide the pAmpscript plasmid. The sequence of the polylinker was 5'—GGATCCGGTACCGCCCGGGCTCTAGAATCGATGTATAC  
10 GTCGACGTTTAAACCATATG—3' (SEQ ID No.:\_\_\_\_).

DNA fragments comprising the adenovirus type-2 E2a and VA RNA sequences were cloned directly into pAmpscript. In particular, a 5962 bp *Srf*I-*Kpn*I(partial) fragment containing the E2a region was  
15 cloned between the *Srf*I and *Kpn*I sites of pAmpscript. The 5962 bp fragment comprises base pairs 21,606-27,568 of the adenovirus type-2 genome. A 732 bp *Eco*RV-*Sac*II(blunted) fragment containing the VA RNAs was cloned into the *Bst*1107I site of pAmpscript. The  
20 732 bp fragment is equivalent to base pairs 10,423-11,155 of the adenovirus type-2 genome.

The DNA comprising the adenovirus type-2 E4 sequences had to be modified before it could be inserted into the pAmpscript polylinker.  
25 Specifically, PCR mutagenesis was used to replace the E4 proximal, adenoviral terminal repeat with a *Srf*I site. The location of this *Srf*I site is equivalent to base pairs 35,836-35,844 of the adenovirus type-2 genome. The sequences of the oligonucleotides used in  
30 the mutagenesis were: 5'—AGAGGCCCGGGCGTTTTAGGGCGGA GTA ACTTGC—3' (SEQ ID No.:\_\_\_\_); and 5'—ACATACCCGCAGGCGTAGAGAC—3' (SEQ ID No.:\_\_\_\_). A 3,192 bp E4 fragment, produced by cleaving the above-described modified E4 gene with *Srf*I and *Spe*I, was  
35 ligated between the *Srf*I and *Xba*I sites of pAmpscript which already contained the E2a and VA RNA sequences to result in the pladenos 5 plasmid. The 3,192 bp



fragment is equivalent to base pairs 32,644-35,836 of the adenovirus type-2 genome.

## 2. rAAV Virion Production Assay:

5                   293 cells were plated in 10-cm tissue culture dishes at  $1 \times 10^6$  cells/dish, and were cultured at 37 °C to reach approximately 90% confluency over a period of from about 24 to 48 hours prior to co-transfections. All transfections were  
10 carried out as described above in Example 1.

                  The 293 cell cultures were transfected with 5 µg of the pVlacZ vector plasmid, 5 µg of plasmid pW1909, and 5 µg of either pladeno 1 or pladeno 5. After the transfections, the media was replaced, and  
15 the cultures were incubated at 37 °C for approximately 72 hours before harvest.

                  Cells from each experimental group were then collected, media was removed by centrifugation (1000 x g for 10 min.), and a 1 mL lysate was produced using 3  
20 freeze/thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made free of debris by centrifugation (10,000 x g for 10 min). rAAV lacZ virion production was then assessed using the techniques described in Example 1. The  
25 results from the experiment are depicted below in Table 9

Table 9	
Accessory Function Vector	rAAV Titer†
pladeno 1	$2.2 \times 10^9$
pladeno 5	$5.5 \times 10^9$

† As determined by the lacZ assay.

35                   As can be seen by the results reported in Table 9, greater rAAV virion production efficiency was seen when using pladeno 5 (pladeno 5 production

yielded 2.5 fold more rAAV virions than pladen0 1 production).

Accordingly, novel accessory functions capable of supporting efficient recombinant AAV virion  
5 production have been described. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined  
10 by the appended claims.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the American Type  
15 Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, under the provisions of the Budapest Treaty. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available  
20 during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. Upon the granting of a patent in this application, all restrictions imposed by the depositor on the availability to the  
25 public of the deposited biological material will be irrevocably removed with the following one exception, as specified in 37 C.F.R. §1.808(b), said exception being that depositor reserves the right to contract with the depository to require that samples of the  
30 deposited material be furnished only if a request for a sample, during the term of the issued patent, meets any one, or all, of the following three conditions:  
(1) the request is in writing or other tangible form and dated; and/or (2) the request contains the name  
35 and address of the requesting party and the accession number of the deposit; and/or (3) the request is communicated in writing by the depository to the

depositor along with the date on which the sample was  
furnished and the name and address of the party to  
whom the sample was furnished. Moreover, the  
designated deposits will be maintained for a period of  
5 thirty (30) years from the date of deposit, or for  
five (5) years after the last request for the deposit;  
or for the enforceable life of the U.S. patent,  
whichever is longer. Should a culture become  
nonviable or be inadvertently destroyed, or, in the  
10 case of plasmid-containing strains, lose its plasmid,  
it will be replaced with a viable culture(s) of the  
same taxonomic description.

These deposits are provided merely as a  
convenience to those of skill in the art, and are not  
15 an admission that a deposit is required. The nucleic  
acid sequences of these plasmids, as well as the amino  
sequences of the polypeptides encoded thereby, are  
controlling in the event of any conflict with the  
description herein. A license may be required to  
20 make, use, or sell the deposited materials, and no  
such license is hereby granted.

	<u>Strain</u>	<u>Deposit Date</u>	<u>ATCC No.</u>
	pBSII-VA RNAs	October 30, 1996	98233
25	p3.3cE4ORF6	October 30, 1996	98234
	p3.3cE2A	October 30, 1996	98235
	pGN1909	July 20, 1995	69871

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Claims:

1. A nucleic acid molecule which provides one or more accessory functions for supporting  
5 recombinant AAV (rAAV) virion production in a suitable host cell and that lacks at least one adenoviral late gene region, said molecule comprising a nucleotide sequence selected from the group consisting of (i) a sequence that provides adenovirus VA RNAs, (ii) an  
10 adenovirus E4 ORF6 coding region, (iii) an adenovirus E2a 72 kD coding region, and any combination of nucleotide sequences (i), (ii) and (iii).

2. A nucleic acid molecule which provides  
15 accessory functions for supporting efficient recombinant AAV (rAAV) virion production in a suitable host cell and that lacks at least one adenoviral late gene region.

20 3. The nucleic acid molecule of claim 2, characterized in that said molecule lacks adenoviral early gene regions 2b and 3.

4. The nucleic acid molecule of claim 2 or  
25 3 which provides accessory functions for supporting efficient rAAV virion production in a human 293 host cell.

5. The nucleic acid molecule of claim 4,  
30 comprising:  
a first nucleotide sequence that provides an adenovirus VA RNA;  
a second nucleotide sequence comprising an adenovirus E4 coding region containing the ORF 6; and  
35 a third nucleotide sequence comprising an adenovirus E2a coding region.

6. The nucleic acid molecule of claim 2, characterized in that said molecule provides accessory functions for supporting efficient recombinant AAV (rAAV) virion production in a suitable host cell that is not infectable by adenovirus or is not capable of supporting adenovirus replication.

7. The nucleic acid molecule of claim 6, comprising:

10 a first nucleotide sequence that provides an adenovirus VA RNA;

a second nucleotide sequence comprising an adenovirus E4 coding region containing the ORF 6;

a third nucleotide sequence comprising an adenovirus E2a coding region; and

15 a fourth nucleotide sequence comprising the adenovirus E1a and E1b coding regions.

8. The nucleic acid molecule of claim 4 or 6, comprising one or more nucleotide sequences derived from an adenovirus type-2 or type-5 genome.

9. An accessory function vector comprising the nucleic acid molecule of any of claims 1, 2 or 6.

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10. The accessory function vector of claim 9, characterized in that said vector is a plasmid.

11. The accessory function vector of claim 10 further comprising a selectable genetic marker.

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12. The accessory function vector of claim 11, characterized in that the selectable genetic marker comprises an antibiotic resistance gene.

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13. The accessory function vector of claim 10 further comprising at least one heterologous promoter region.

5 14. The accessory function vector of claim 13, characterized in that the at least one heterologous promoter region comprises a nucleotide sequence substantially homologous to the cytomegalovirus (CMV) immediate early promoter region.

10 15. An accessory function system for recombinant AAV (rAAV) virion production, comprising first, second, and third accessory function vectors, wherein:

15 (a) said first accessory function vector comprises a nucleotide sequence that provides adenovirus VA RNAs;

(b) said second accessory function vector comprises an adenovirus E4 ORF6 coding region; and

20 (c) said third accessory function vector comprises an adenovirus E2a 72 kD coding region.

16. The accessory function system of claim 15 characterized in that the first accessory function vector is the plasmid pBSII-VA RNAs (ATCC Accession Number 98233), the second accessory function vector is p3.3cE4ORF6 (ATCC Accession Number 98234), and the third accessory function vector is p3.3cE2A (ATCC Accession Number 98235).

30 17. A host cell that has been transfected with the accessory function vector of claim 9.

35 18. A cell capable of producing recombinant AAV (rAAV) virions when transfected with an AAV vector, said cell comprising the host cell of claim 17 co-transfected with an AAV helper construct that is

capable of being expressed in said cell to provide AAV helper functions.

19. A method of producing recombinant AAV (rAAV) virions, comprising:

- (a) introducing an AAV vector into a suitable host cell;
- (b) introducing an AAV helper construct into the host cell, said helper construct comprising AAV coding regions that are expressed in the host cell to complement AAV helper functions missing from said AAV vector;
- (c) introducing the accessory function system of claim 15 into the host cell, said accessory function system providing accessory functions for supporting efficient rAAV virion production in the host cell; and
- (d) culturing the host cell to produce rAAV virions.

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20. A method of producing recombinant AAV (rAAV) virions, comprising:

- (a) introducing an AAV vector into a suitable host cell;
- (b) introducing an AAV helper construct into the host cell, said helper construct comprising AAV coding regions that are expressed in the host cell to complement AAV helper functions missing from said AAV vector;
- (c) introducing the accessory function vector of claim 9 into the host cell, said accessory function vector providing accessory functions for supporting efficient rAAV virion production in the host cell; and
- (d) culturing the host cell to produce rAAV virions.

21. A recombinant AAV virion produced by  
the method of claim 19 or 20.

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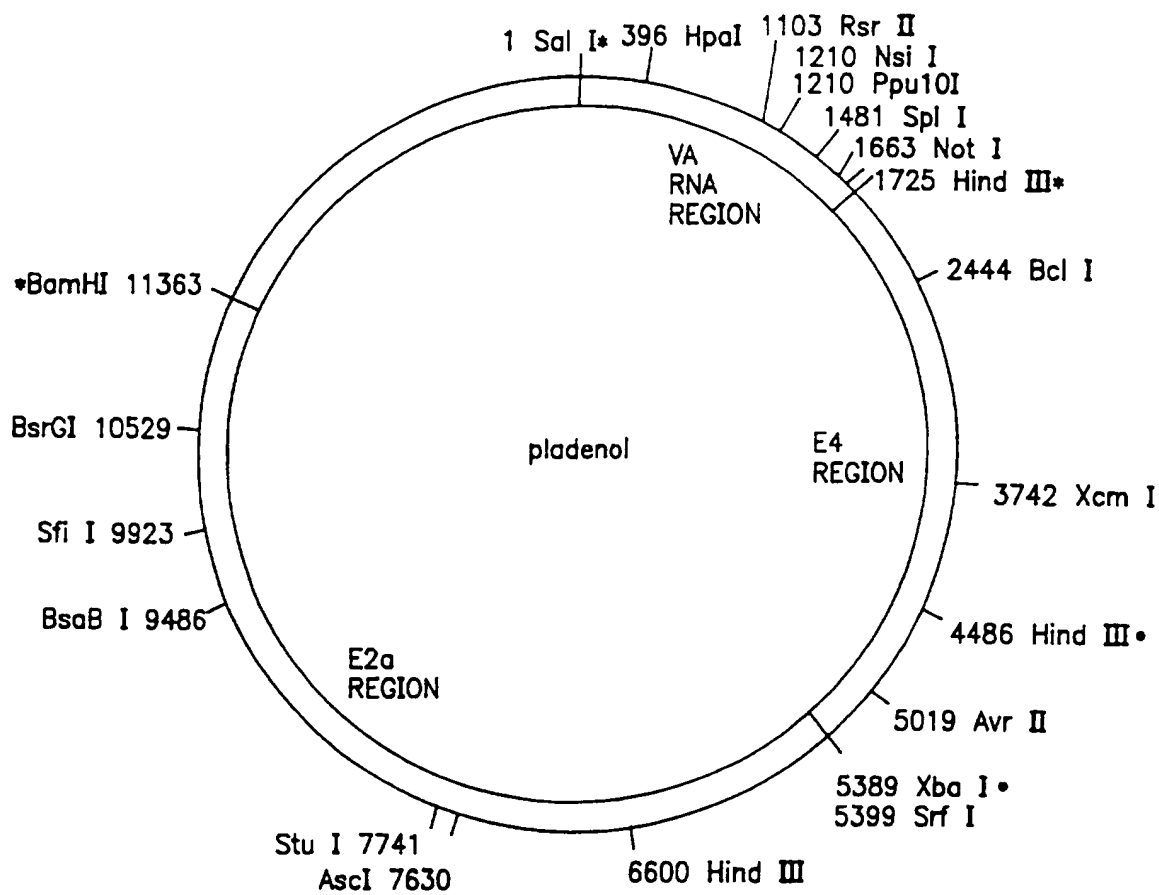
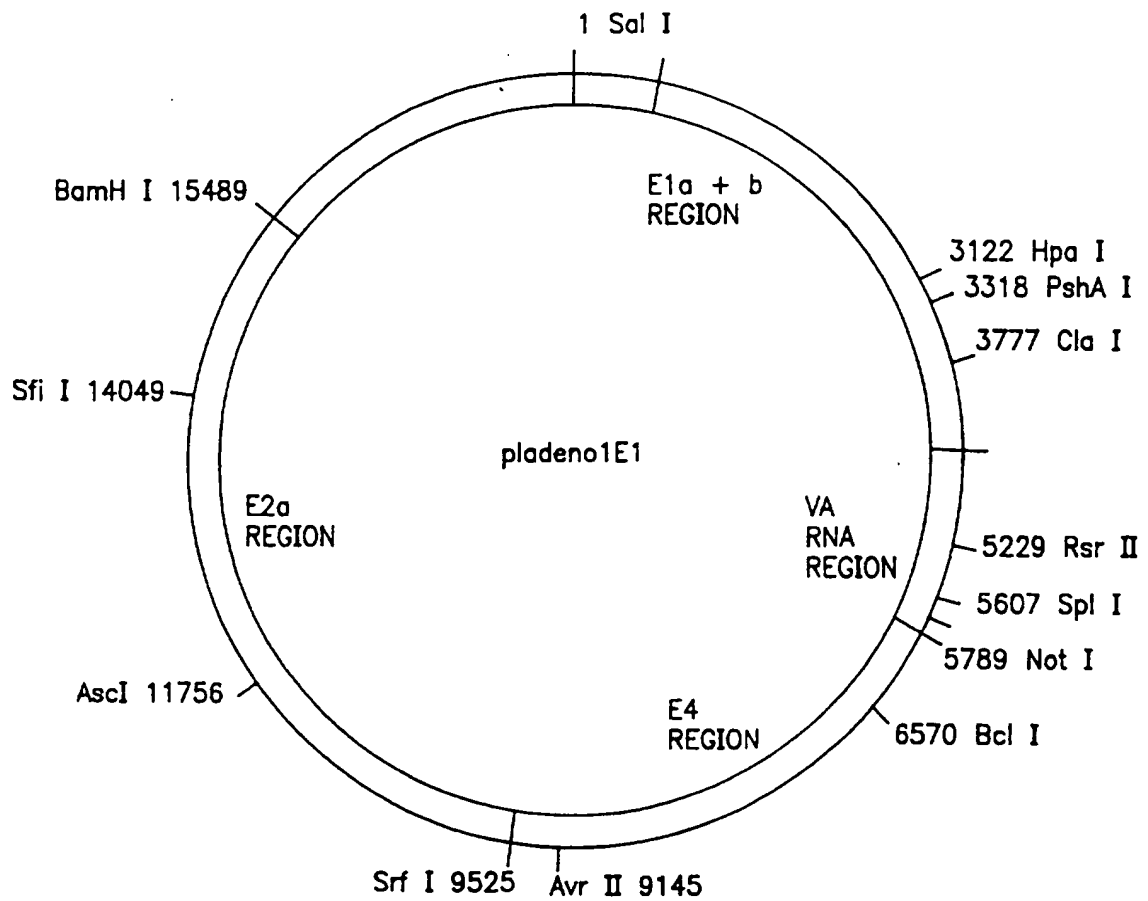


FIG. 1

2 / 3

**FIG. 2**

3 / 3

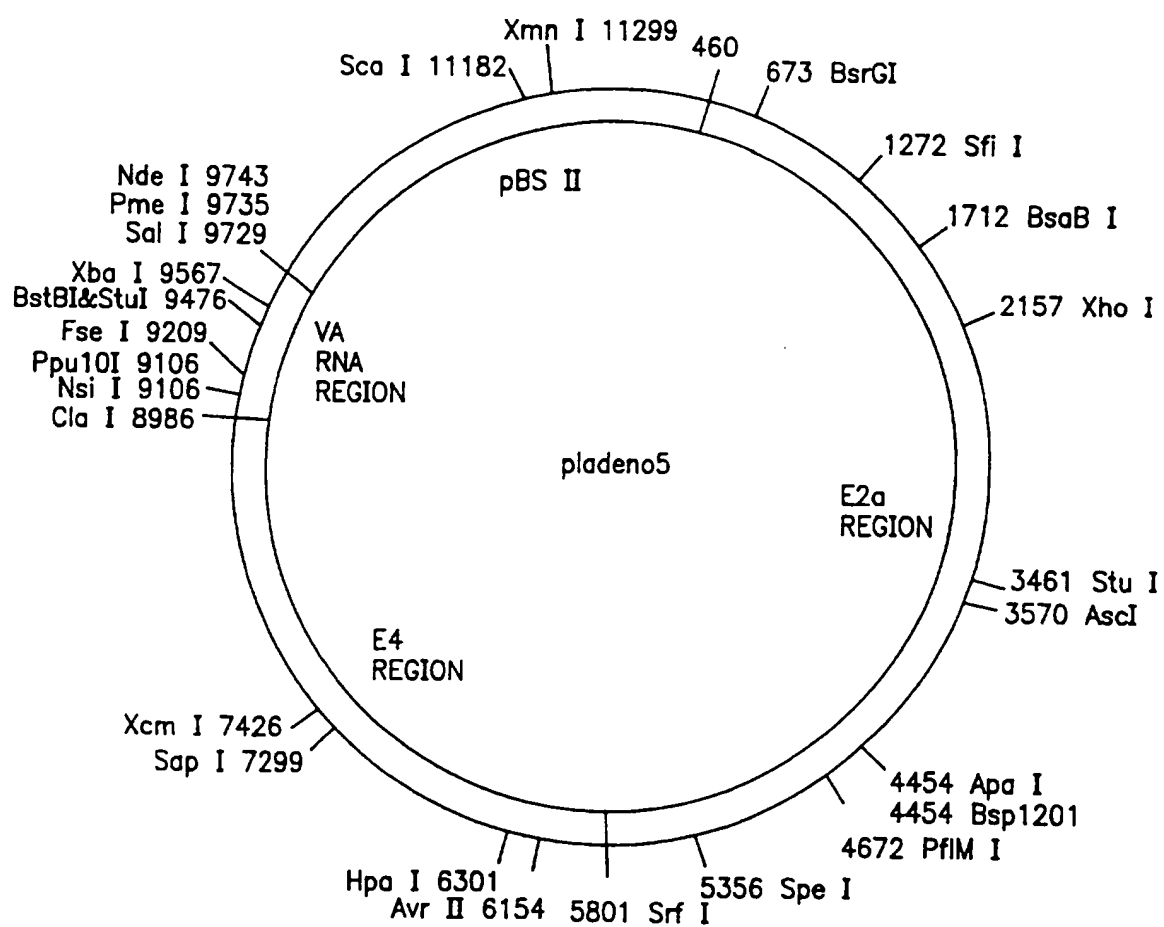


FIG. 3

PCT/US 96/18089

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C07K14/075 C12N7/04 C12N5/10 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 78, no. 3, March 1981, pages 1925-1929, XP000614746 JANIK J E ET AL: "LOCATIONS OF ADENOVIRUS GENES REQUIRED FOR THE REPLICATION OF ADENOVIRUS-ASSOCIATED VIRUS" see the whole document ---	1-4, 8-10, 13, 17, 18
X	VIROLOGY, vol. 126, no. 2, 30 April 1983, pages 505-516, XP000614629 CARTER B J ET AL: "PROPERTIES OF AN ADENOVIRUS TYPE 2 MUTANT, AD2D/807, HAVING A DELETION NEAR THE RIGHT-HAND GENOME TERMINUS: FAILURE TO HELP AAV REPLICATION" cited in the application see the whole document ---	1-3, 8, 9, 13, 17, 18

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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search  28 February 1997	Date of mailing of the international search report  12.03.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer  Chambonnet, F

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A	JOURNAL OF VIROLOGY, vol. 41, no. 3, March 1982, pages 868-876, XP000614630 LAUGHLIN C A ET AL: "EFFECT OF DELETIONS IN ADENOVIRUS EARLY REGION 1 GENES UPON REPLICATION OF ADENO-ASSOCIATED VIRUS" cited in the application see the whole document ---	1,4,7
A	FR 2 707 664 A (CENTRE NAT RECH SCIENT ;ROUSSY INST GUSTAVE) 20 January 1995 see claims 1-4,17,18,21-24; example 4 ---	1-21
X,P	BLOOD, vol. 86, no. 10, SUPPL. 01, 15 November 1995, page 627A XP000614696 COLOSI P ET AL: "AAV VECTORS CAN BE EFFICIENTLY PRODUCED WITHOUT HELPER VIRUS" see abstract ---	1-21
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